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13. ABSTRACT (Maximum 200 Words)

Representational difference analysis method was used to isolate metastasis-specific genetic markers for breast cancers that are prone to develop metastasis. Of the 15 candidate MADS that have been isolated, 3 were found to be promising markers for breast metastasis. Screening of MADS-IX on a tumor cell line with homozygous loss of PTEN gene, revealed that MADS-IX is neither a part of PTEN gene nor localized in the homozygous loss region of chromosome 10q arm encompassing PTEN. Our Fluorescence in situ hybridization screening for MADS on primary tumor tissue touch preparation cells was successful. The results indicate that the MADS-IX could be used as a FISH probe for distinguishing the primary tumors that did and that did not develop metastasis. A tumorigenesis cum metastasis SCID mouse model has been developed and was successfully used to evaluate the metastatic potential of MDA-MB-435 breast carcinoma cells transfected with a metastasis suppressor gene (nm23) and a metastasis promoter gene (ErbB2). After the evaluation of these were subjected to cDNA microarray studies which resulted in the discovery of several known/novel genes associated with nm23 mediated metastasis inhibition and ErbB2 mediated metastasis promotion and identification of certain drug targets.

14. SUBJECT TERMS
Breast Cancer, Metastasis, Representational difference analysis (RDA), Laser capture microdissection (LCM), Metastasis associated DNA sequences (MADS), Fluorescence in situ hybridization (FISH)

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(4) Introduction

The clinical outcome is generally positive for patients with node-negative breast carcinoma (group-I) who have been treated with surgery or surgery and radiation therapy. In about 13% of the patients (Group-II), however, the disease recurs, and they are at risk of death. The aim of this project is to develop a panel of molecular genetic markers for detecting the group II mammary carcinomas that are prone to developing metastases so that they could be treated more aggressively. There will also be considerable benefit to the women with group-I mammary carcinomas that are not likely to metastasize (an estimated 87%). These women could then be treated more conservatively, sparing them the considerable physical, mental and financial costs of the treatment and with greater ease of mind than is possible today. Genetic tests capable of identifying patients at risk for metastatic spread and/or better treatment targeted to eradicate metastatic tumor deposits could have a dramatic impact on the overall survival of these patients. The technical objective of this project is to isolate DNA sequences and/or genes whose loss of wild-type function represents a step in the acquisition of metastatic property by ductal mammary carcinoma cells. To accomplish this objective we have used representational difference analysis (Lisitsyn et al. 1993) on normal/primary and metastatic archival tissue samples from patients with breast carcinoma. These genetic markers could be useful for differentiating with high sensitivity and reliability ductal mammary carcinomas that are prone to developing metastases from those that will not. In addition, further knowledge of the genes and genetic mechanisms that play an important role in metastasis could ultimately lead to the development of improved therapeutic procedures.

(5) Body

Statement of Work (3 years)

Task. 1: Isolation of differential sequences specific to breast metastasis using RDA.

i. Collection of additional tissues of ductal mammary carcinoma (DMC) and continuation of ongoing RDA product characterization (Months 1-12)
ii. Confirmation of histopathology of tissues before LCM (Months 1-24)
iii. LCM of 30 cases of DMC tissues with normal, primary and metastasis cell components (Months 2-30)
iv. RDA of 30 cases of DMC (Months 1-14)

Task 2.: Characterization of the differential RDA products.

i. Cloning of RDA products isolated from the loss side (Months 1-18)
ii. Sequencing and Southern blot analysis of the clones (Months 1-20)
iii. Homology search of positive clones (Months 2-28)
iv. Northern blot analysis of the significant clones from the past and present RDA experiments (Months 6-25)
v. Isolation of complete gene sequences (Months 6-25)

Task. 3: Further analysis of candidate genes of metastasis.

i. Screening the metastasis-specific genes on at least 120 DMC cases and statistical analysis (Months 12-30)
ii. *In vitro* and *in vivo* functional assays of metastasis (Months12-30)
iii. Antisense oligonucleotide-mediated disruption of mRNA translational studies (months 18-34)
iv. Compilation of data for the submission of final grant report (Months 33-36).

Final Year Progress Report:

We have undertaken all experiments that were proposed under each task and following is the progress of the work done so far. Please note that we now named markers associated with metastasis as Metastasis Associated DNA Sequences [MADS] instead of Metastasis Associated Gene Sequences [MAGS].

Task 1:

In order to isolate metastasis associated DNA sequences (MADS) we have used genomic representational difference analysis (RDA), a subtractive hybridization method (Lisitsyn et al., 1993; Li et al., 1997), on human breast carcinoma tissue samples (normal, primary and metastatic tumor tissues) and cell lines (low and high metastatic cells; tumor cells derived from human primary tumors and metastatic lungs developed in a mouse model). From 11 additional RDA experiments, several hundred clones were isolated. Using "total probe" method, 4 additional clones different from the previously isolated 11 MADS, were identified. Thus bringing the total number of unique candidate metastasis associated DNA sequences (MADS) to 15. The tissue sections of 12 out of 20 selected cases were found suitable for LCM (Emmert-Buck et al. 1996). Except for the first RDA, all the remaining RDA experiments were performed by comparing DNA samples recovered from primary and metastatic tumor cells. We could not complete RDA of the remaining 18 cases (as proposed) so far because of two difficulties. Firstly, each RDA experiment followed by cloning and characterization of the differential products has taken much of our time. Secondly, we found that some of the archival samples are not suitable for LCM. Though we were successful in performing Single Cell Microdissection (SCM) on some of the cases, unfortunately we found that the DNA isolated from these microdissected cells was degraded and not suitable for Representational Difference Analysis (RDA) experiments.

Task 2:

To further characterize the candidate metastasis-specific sequences obtained from the first set of RDA experiments, we did homology search and also localized them on human chromosomes by using GeneBridge 4 Radiation Hybrid panel (Research Genetics, Inc.).

RH mapping is a somatic cell hybrid technique that was developed to construct high-resolution contiguous maps of mammalian chromosomes. The distance provided by this method is directly proportional to the physical distance. RH mapping uses a statistical program (RHMAP) that will provide the best map along with a measure of the relative likelihood of one order versus another (http:www.sph.umich.edu/group/statgen/software). The RH panel of 93 radiation hybrid clones represent the whole human genome. This panel is a subset of 199 clone panel developed by Walter et al (1994). A human cell line was exposed to 3000rad of X-rays and then fused with thymidine-deficient hamster recipient cell, creating a panel of hybrids with around 1000 kb resolution. of sequences by Radiation Hybrid mapping (**http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper**). The sequence homologies and RH mapping results of the MADS were presented in Table 1

Table 1. The length, homology search and RH mapping results of the metastasis associated DNA sequences (MADSs).

MADSs	Length (bp)	Homology	RH mapping
I	205	Chromosome 5 (97%) AC005915.1	Not successful
II	144	Chromosome 21 (99%) AC010463.6	Not successful
III	277	Chromosome 1 (97%) AL359265.8	Not successful
IV	185	Chromosome 5 (98%) AC124857.2	Chromosome 5; Places 19.72 cR from WI-6737
V	220	Chromosome 16 (98%) AC004234.1	Not successful
VI	242	Chromosome 7 (97%) AC09333.1	Not successful
VII	190	Chromosome 6 (98%) AL121935.17	Chromosome 6; Places 1.51 cR from WI-3110
VIII	170	Chromosome 6 (82%) AC013429.12	Chromosome 6; Places 3.67 cR from AFMA191WD1
IX	180	Chromosome 10 (94%) AC022541.10	Chromosome 10; Places 5.66 cR from D10S546
X	192	Chromosome 15 (98%) AC104260.5	Chromosome 15; Places 1.71 cR from D15S157

All the groups except MADS-XI (TG repeat sequence group) were subjected to RH mapping and analyzable data was obtained for only 5 groups. Sequences under groups VII and VIII were localized on chromosome 6 and those under groups IV, IX and X were localized on chromosomes 5^{th}, 10^{th} and 15^{th} respectively. It is interesting to note that one of these putative metastasis-specific sequences (MADS- IX) is localized on chromosome 10^{th}, close to an important tumor/metastasis suppressor gene, the PTEN (Li et al. 1997). This increases the likelihood that this sequence plays an important role in breast cancer metastasis.

Additional MADS:

The following 4 new MADS have been identified from additional RDA experiments using "Total Probe" method:

MADS-XII: Product size: 125bp; 96% homology with BAC clone RP11-651C2 on chromosome 4.
MADS-XIII: Product size: 212bp; 99% homology with BAC clone RP11-452C13 on chromosome 7.
MADS-XIV: Product size: 231bp; 92% homology with BAC clone AC000119 on chromosome 7.
MADS-XV: Product size: 172bp; 95% homology with BAC clone AL590825 on chromosome 6.

Task 3:

To determine if these MADS are indeed associated with metastasis, DNA samples were used from cells recovered with the SCM and LCM methods from normal, primary and metastatic tissue samples from additional patients. These DNA samples were screened with different MADS by using Southern blot/PCR/FISH methods.

Further characterization and screening of MADS-XI on additional patient samples:

This has an interesting and also novel pattern of TG/AC repeats (Fig.1). Since dinucleotide repeats (TG/AC) are correlated in several cancers with the severity of the disease, we became interested to screen some breast cancer patient samples. PCR screening of patient samples was not possible due to TG repeats. Consequently we used the sequence as a α^{32}p-dCTP probe to screen a Southern blot consisting of normal, primary and metastatic cell DNA of 5 patient samples (Fig. 2). The results showed that MADS-XI was missing in the metastatic cell DNA of 4 out of 5 patient samples indicating its importance in metastasis. These results were published in "Cancer Detection and Prevention" (Mukherjee et al 2003).

***V*GATCTTAATCCGGGGAGTGGCGTATGTAGTAGAAGAGTCTGGATTTGAGTAGT GTATGGTAACGCCAG (T-G)$_5$ C (T-G)$_4$GTA (T-G)$_2$G (T-G)$_4$T(T-G)3 GCA(T-G)$_3$G(T-G)$_2$TATGG(T-G)$_3$G (T-G)$_3$A (T-G)$_4$GTGCG(T-G)$_2$AGATACGTGG(T-G)$_3$GGG(T-G)$_6$ G (T-G)$_3$ G (T-G)$_5$ GTA (T-G)$_2$ G (T-G)$_3$ G *V*.**

Fig. 1. Complete MADS-XI with unique sequence (bold letters) and enriched with TG repeats; *V*= vector sequence

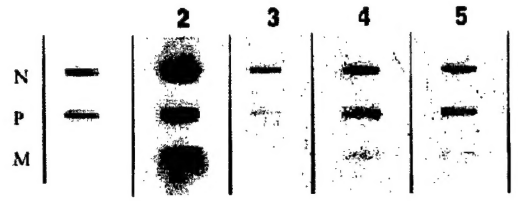

Fig. 2. Results of slot blots using 32P-CTP probes prepared from the clone #41 (MADS-XI) on normal (N), primary (P) and metastatic (M) genomic DNA samples from 5 ductal breast carcinoma patients. In cases 1, 3, 4, and 5, the sequence 41 hybridized to normal and primary but not to metastatic cell DNA, probably indicating the loss of this sequence in metastatic cell DNA of these cases. Case 2 however showed signals in N/P/M samples.

Further characterization and screening of MADS-IV on additional patient samples:

To determine if the MADS obtained from our RDA experiments are parts of functional genes, RT-PCR experiments were conducted. Primers were designed from different MADS and used on normal human total RNA. MADS-IV showed expression of the transcript (Fig. 3).

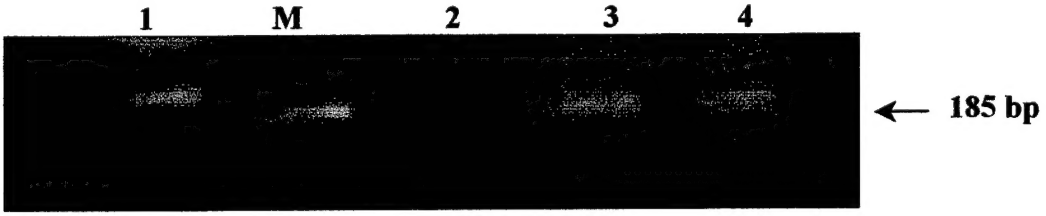

Fig. 3. RT-PCR of normal RNA using MADS-IV primers showing that the gene is expressing. Lane M: DNA marker band; Lane 1: Positive control; Lane 2: Negative control; 3-4: Total RNA from normal human tissue digested with DNAse I.

RT-PCR results showed that MADS–IV is an expressed sequence (Fig. 3). To confirm this, we screened multiple tissue RNA Master Blots™ (CLONTECH Laboratories, Inc.) using MADS-IV as a α32p-dCTP probe. Results revealed that MADS-IV expresses in mammary gland and also in all the other human tissues, but not in non-human RNA or DNA samples (E. coli synthetic poly r(A), yeast tRNA or total RNA, or C_0t1 DNA), indicating that MADS-IV is a part of a human gene that transcribes commonly in human tissues (Fig. 4). This reinforces the fact that this MADS is part of a functional gene possibly involved in breast tumorigenesis/metastasis.

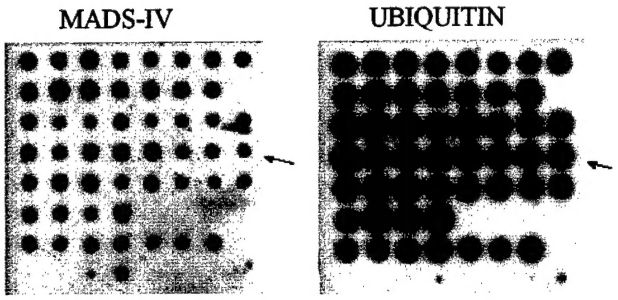

Fig. 4. Results of Northern hybridization of RNA Master Blot (CLONTECH Laboratories, Inc.) using MADS-IV as probe. It showed expression in all of the human tissues, but not in non human RNA or cDNA (synthetic poly r(A), yeast tRNA or total RNA, or C_0t1 DNA) indicating that it is an expressed human gene sequence. No significant change in the intensity of signal was noticed in breast tissue (Arrow) compared to the remaining tissues. Ubiquitin gene was used as an internal control for normalization.

While screening MADS-IV on DNA samples of cell lines derived from normal tissue and primary tumors of three breast carcinoma patients who had known losses in specific chromosomal regions, we found that MADS-IV was missing in the tumor DNA sample of one patient cell line (Fig. 5). PCR screening of MADS-IV in additional 9 primary tumor DNA samples with matched normal tissue DNA samples, received from Dr. Wahab (Cairo University), we observed the loss of MADS-IV in 2 tumor samples (Tumors 6 and 9). The intensity of the PCR product for MADS-IV was less than half in tumor 6 compared to normal, while it is completely missing in tumor 9, which indicates that MADS-IV probably is heterozygously lost in tumor 6 and homozygously lost in tumor 9 (Fig. 6). Thus far MADS-IV was found to be missing in 3 tumors out of 12 tumors screened. These findings are in the process for communication.

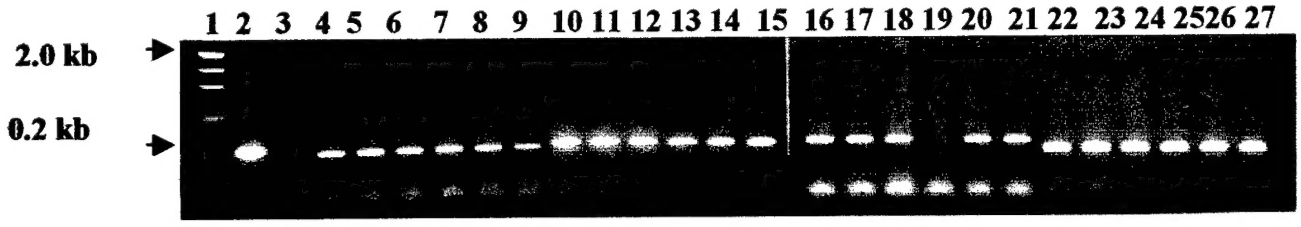

Fig. 5. PCR screening of 4 MAGS on DNA samples of paired normal cells (lanes with even numbers from 4-26) and primary tumor cells (lanes with odd numbers starting from 5-27) derived from three breast carcinoma patients with loss of heterozygosity for a region on chromosome 10q encompassing gene PTEN. Lanes 1: 2Kb DNA marker; 2:

Positive control; 3: Negative control; PCR results with MAGS X with product size of 148bp (lanes 4-9); MAGS VIII with product size of 171bp (lanes 10-15); MAGS IV with product size of 185bp (lanes 16-21) and MAGS II with product size of 144bp (22-27). Lane 19 represents tumor cell line sample of a patient showing absence of a 185 bp product. G3PDH internal controls are not shown in the figure.

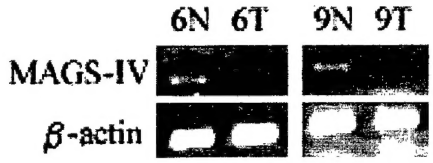

Fig. 6. PCR screening of MADS-IV on normal (N) and tumor (T) DNA samples. Tumor 6 showed heterozygous loss and tumor 9, showed homozygous loss of MADS-IV.

Further characterization and screening of MADS-IX on additional patient samples:

The RH mapping revealed that MADS-IX is localized to a 21cR interval between markers, D105539 and D10S549, corresponding to human chromosome 10 band q21.1. To determine if this gene is close to PTEN, we screened 4 tumor cell lines and matched normal DNA (the first 3 tumor cell lines had known losses in specific chromosomal regions, the 4th cell line had loss of homozygosity of PTEN gene). PCR screening of MADS-IX and PTEN showed that MADS-IX is present in all the 4 tumor cell lines (HCC-1806, HCC-1143, HCC-1428 and HCC-1937), especially the 4th cell line. PTEN is present in 3 cell lines, but missing in the 4th cell line, indicating that it is a novel DNA sequence but is neither a part of PTEN gene nor localized in the homozygous loss region of chromosome 10q arm encompassing PTEN region (Fig. 7).

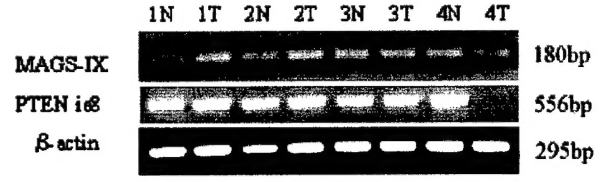

Fig. 7. PCR screening of MADS-IX on normal and tumor DNA samples from breast carcinoma cell lines that losses in specific chromosomal regions (especially the 4th which had loss of homozygosity of PTEN gene), showed the presence of MADS-IX in all 4 cell lines. The second row showing the presence of PTEN in the normal cell DNA but missing in the tumor cell DNA of 4th cell line (4T lane), indicating that MADS-IX is neither a part of PTEN nor localized in the loss region of 10q which encompasses the PTEN gene. β-actin was used as an internal control.

So far we have been using PCR (Southern slot-blot, where not possible) methods for the characterization of all the MADS on microdissected normal, primary and metastatic tumor cell DNA samples. One of the main problems we encountered was the inconsistency in the PCR results. We had to repeat the PCR experiments several times to

confirm the absence/presence of the MADS (partial or complete) in a given patient sample. The partially appearing PCR bands are most of the times confusing because they could be partial loss or a possible contamination from few stromal (normal) cells during microdissection. We therefore proposed to screen the patient samples by FISH. This method we believe is more reliable method to differentiate the archival tumors that did metastasize from those which did not. Since normal and tumor cells are present side by side in a primary tumor sample, we proposed that these markers should be present in normal cells and missing (one allele or 2 alleles) in the tumor cells. In these attempts we used MADS-IX as a FISH probe. Generally, FISH requires larger sized probes (DNA fragment: 1-1000kb). Since the size of the MADS-IX is only 180bp, we designed primers from the BAC clone that has homology with MADS-IX, and isolated a DNA fragment sized 2 Kb encompassing the MADS-IX by long-accurate PCR using normal human genomic DNA as the template. This 2 Kb MADS-IX is used as a FISH probe to localize on human metaphase chromosomes to determine that MADS-IX is a human sequence and not an artifact. We labeled MADS-IX with spectrum green (Vysis) and probed on human metaphase chromosomes (Fig. 8). The results showed that MADS-IX is localized on chromosome10 at q21 region (close to centromere on the long arm).

Fig. 8. Localization of MADS-IX on human metaphase chromosomes. Centromere of chromosome 8 was used as a positive control probe. MADS-IX was found to localize around 10q21 chromosome region (close to centromere). Both the MADS-IX and CEP-8 are labeled with spectrum green (Vysis).

To determine if this MADS-IX is indeed associated with metastasis, DNA was used from cells recovered with the LCM method from normal, primary and metastatic tissue samples from 5 additional patients. These samples were screened by PCR using primers designed for MADS-IX. As shown in the figure 9, MADS-IX was present in normal cell DNA samples of all the 5 patients but the intensity of signal was less in the primary tumor cell DNA samples from 2 patients and totally undetectable in the metastatic cell DNA of these two patient samples, suggesting a loss of this gene sequence during progression to metastasis. This analysis revealed that MADS-IX was lost in the transition from normal to primary to metastasis in 2 of 5 cases (3 of 6 cases if the case used in the RDA assay is included). The fact that it was present in primary tumor cells, but missing in lymph node metastatic cell foci strongly suggests that this is a marker for a novel metastasis suppressor gene. These findings were published in "Breast Cancer Research" (Achary et al 2003).

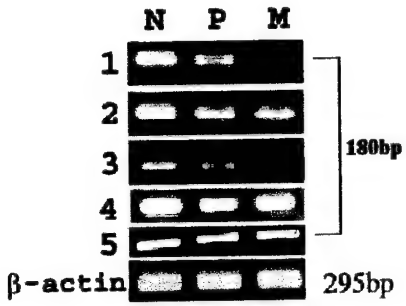

Fig. 9. PCR screening of MADS IX on normal, primary and metastatic cell DNA samples of 5 patients. PCR results showing the target DNA band (180bp) missing in the metastatic cell DNA of patients 1 and 3 (M lane). β-actin as an internal control.

Identification of loss of MADS-IX on primary tumors by FISH:

Since our long term goal is to screen the MADS as FISH probes on primary tumor tissue sections to predict if that primary tumor is prone to developing metastasis or not, we made an attempt to screen MADS-IX as a FISH probe on normal tissue and primary tumor tissue sections of a patient who developed metastasis. We labeled chromosome 8 centromere (positive control) with orange red and MADS-IX with orange green and screened normal tissue, primary tumor and positive lymph node tissue sections. Normal tissue section showed several red and green signals but the cell/nuclear morphology was not clear due to lot of fat around the cells. In the primary tumor tissue sections, as expected the tumor cell nuclei were much larger than the normal cell nuclei and the FISH signals were clearer than that of the normal cell nuclei. Since we used 2 probes, we expected 3 patterns of labeling signals in the nuclei (presuming the centromere of chromosome 8 is not lost in these tumors), (i): 2 reds and 2 greens, if it is a normal cell nucleus or a tumor cell nucleus that did not lose MADS-IX; (ii): 2 reds and 1 green, if there is heterozygous loss of MADS-IX; (iii): 2 reds and no green, if there is homozygous loss of MADS-IX. As shown in figure 10, we observed clearly all the three patterns in the primary tumor section. Out of 50 cells observed in the primary tumor section, 26 clearly were of first pattern (normal), 20 were of second pattern and 3 of third pattern. Similar FISH in the positive lymph node tumor section of the same primary tumor showed more pattern 3 cells rather than pattern 2 cells. On the other hand in the primary tumor that did not develop metastasis, out of 50 cells observed, interestingly we did not record any cell that belonged to pattern 3 and only 3 cells that showed pattern 2 (loss of heterozygosity). These results are promising but due to lack of proper morphology of cells/nuclei in these tumor tissue sections, we found it difficult to use this strategy satisfactorily (Fig. 10).

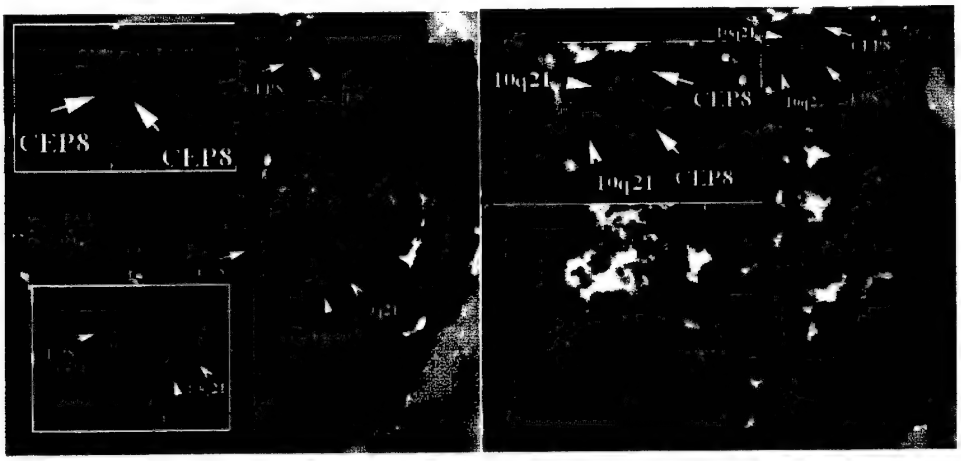

Fig. 10. Localization of MADS-IX in different cells of primary tumor tissue section. Chromosome 8 centromere (positive control with red signals) was labeled with spectrum orange and MADS-IX with spectrum green (green signals). 3 patterns of labeling signals in the nuclei were observed: (i): 2 reds and 2 greens, if it is a normal cell nucleus or a tumor cell nucleus (right side top inset) that did not lose MADS-IX; (ii): 2 reds and 1 green (left lower inset), if there is heterozygous loss of MADS-IX; (iii): 2 reds and no green, if there is homozygous loss of MADS-IX (left top inset).

Touch preparations:

We used a simple FISH procedure namely, touch preparations, to substitute the tumor tissue section method. In this method, the tumor tissue material was exposed by trimming of the paraffin material from the block and then by touching the tumor tissue side at several places on a slide, the cells were attached from the tumor tissue block on to the slide surface. Conventional methods were used to fix the cells followed by standard FISH procedure. We used 3 cases of primary tumors that developed metastasis (positive lymph nodes) and another 3 that did not develop metastasis. As shown in the figure 11, the nuclei and presence/absence of signals are clear and significantly superior than the tumor tissue section method. The results in the following table indicate that the MADS-IX could be used as a promising marker to differentiate Groups I and II primary breast tumors that do and do not develop metastasis respectively.

Group I: (+ LNs): No loss: 54%; Heterozygous loss: 38%; Homozygous loss: 8%
Group II: (- LNs): No loss: 80%; Heterozygous loss: 20%; Homozygous loss: 0%

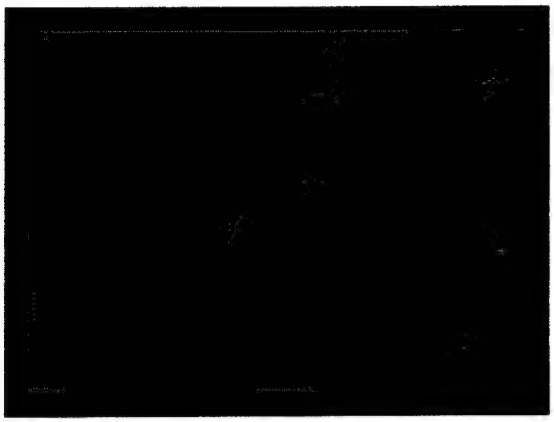

Fig. 11. Collection of cells from different parts of a slide prepared from a primary tumor touch preparation that had positive lymph nodes. MADS-IX was labeled with spectrum orange and cetromere of chromosome 8 (reference) was labeled with spectrum green. While most of the nuclei, show no loss of red signals, a nucleus at 4 O'clock position shows loss of heterozygosity (1 red signal) and a nucleus at 6 O'clock position shows loss of homozygosity (no red signals).

Evaluation of metastatic potential MAGS/MADS:

To evaluate the metastatic potential of a gene, we developed a mammary fat pad (MFP) SCID mouse model (Fig. 12). We used 435 cells transfected with a metastasis suppressor (nm23) gene and a metastasis promoter (ErbB2) gene and successfully examined the primary tumors and lungs in this model. MDA-MB-435 transfected with nm23/ErbB2 and vector only transfected cells were injected (@1X10^6 cells) in a volume of 100 µl of DMEM without serum into the right sub-axillary MFPs of anesthetized 5- to 6-week-old female SCID mice. Five mice each were used for transfected, vector only and parental non-trasfected 435 cells as a control. Tumor sizes were monitored a week after inoculation of tumor cells. When the mean tumor diameter reached 1.0 cm, tumors were surgically removed. Six weeks later the animals were sacrificed and lungs were examined for visible metastatic foci and counted. While mice injected with nm23 transfected cells showed very few metastatic foci in the lungs compared to the control, those injected with cells transfected with ErbB2 showed large number of metastatic foci compared to the control. These results suggest that our mouse model is working satisfactorily in our hands. Animals were maintained under the guidelines of the NIH and approved protocols of IACU Committee of AECOM.

Similarly, we are planning to transfect BAC/MADS into 435 cells and evaluate the metastatic potential of MADS containing BACs. We identified 14 BACS that contain corresponding 14 MADS using gene bank search. The candidate BACs will be retrofitted with a selectable marker *neo* and then introduced individually into metastatic human mammary MDA-MB-435 tumor cells following the procedure described by Mejia and Monaco (1997) to determine their potential in the inhibition of metastasis.

In Vivo metastasis assay

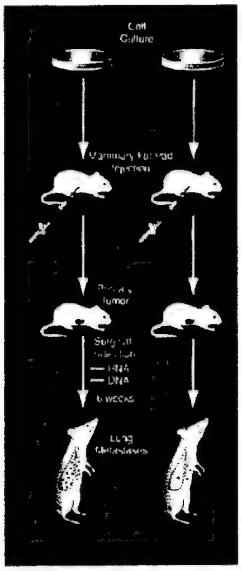

Fig. 12. *In Vivo* Spontaneous Metastasis Model: The left panel represents the mouse injected with control cells (highly metastatic) and right panel with cells transfected with a gene which has the potential to inhibit or reduce metastasis.

(6) Key Research Accomplishments:

- Fifteen candidate metastasis-specific DNA sequences in breast carcinoma have been isolated. Some of them were localized on human chromosomes by RH mapping and FISH methods.
- A candidate sequence, MADS-IV was found to be missing in one of the three breast carcinoma patient tumor cell lines, which had mutations in the gene PTEN.
- RT-PCR and Dot/Northern blot experiments indicated that MADS-IV is an expressed sequence.
- Screening of human multiple tissue Northern blot experiments reinforced the fact that MADS-IV is an expressed sequence. Its expression in human breast tissue RNA besides other tissue RNA samples indicated that the expression of MADS-IV is not breast tissue specific.
- Two additional MADS have been isolated namely, MADS-IX (Achary et al 2003) and MADS-XI (Mukherjee et al 2003) that were found to be missing (partial/complete) in primary/metastatic cell DNA of some patients other than the index cases.
- Screening of MADS-IX on different tumor cell lines (one of them had homozygous loss of PTEN gene) revealed that MADS-IX is an entirely separate genetic segment and is neither a part of PTEN gene nor localized in the homozygous loss region of chromosome 10q arm encompassing PTEN region. Therefore it could be a novel gene sequence on chromosome 10, associated with metastasis (Achary et al 2003).

- Attempts were made to replace PCR method with Fluorescence in situ hybridization (FISH) for screening MADS on primary tumor tissue sections. Screening of MADS-IX in 50 cells from the primary tumor that had positive lymph nodes showed 27 cells with no loss of MADS-IX, 20 cells with loss of heterozygosity and only 3 cells with loss of homozygosity. On the other hand, the primary tumor with negative lymph nodes showed 47 cells with no loss, only 3 cells with loss of heterozygosity and none with loss of homozygosity. These results indicate the potential of the MADS-IX as a FISH probe in identifying the primary tumors that did and did not develop metastasis. However we did not find consistency in the results due to overlapping of cells and unclear cellular/nuclear margins in the tumor tissue sections.

- Similar studies with tumor cell touch preparations using MADS-IX as FISH probe however were satisfactory. While group I tumors with + LNs showed cells with no loss of MADS-IX in 54%; heterozygous loss in 38% and homozygous loss in 8% cells/nuclei, group II tumors with – LNs, on the other hand showed cells with no loss of MADS-IX in 80%; cells with heterozygous loss in 20% and none with homozygous loss. Based on the studies performed using these 6 cases of breast carcinoma, we found that MADS-IX could be used as a candidate DNA marker to differentiate tumors that developed metastasis (positive lymph nodes) from those that did not (negative lymph nodes).

- The tumorigenesis cum metastasis SCID mouse model was developed and used successfully with the MDA-MB-435 breast carcinoma cells transfected with a metastasis suppressor (nm23) and a metastasis promoter (ErbB2) (Zhao et al 2003; submitted). The incidence of low number of lung metastatic foci in lungs in nm23 transfected experiments and incidence of a large number of metastatic foci in the lungs of ErbB2 transfected experiments compared to controls suggest that our mouse model is working satisfactorily.

- After evaluating the metastatic potential of the cell lines transfected with nm23 and ErbB2 genes, we subjected them to cDNA microarray experiments. These experiments revealed several known/novel genes/ESTs that are associated with nm23-directed metastasis suppression (Zhao et al 2003; submitted) and ErbB2 directed metastasis promotion (Xue et al; in preparation) some of which may be used as drug targets.

7) Reportable outcomes:

Manuscripts, abstracts and presentations

Publications

Directly related to the grant:

P. Mohan R. Achary, Zhao H, Fan Z, Gogineni S, Pulijaal V, Herbst L, Mahadevia PS, Jones, J. Klinger HP, Vikram B. 2003. A candidate metastasis associated DNA marker for ductal mammary carcinoma. **Breast Cancer Research** 5: 52-58. (Annexure I)

B. Mukherjee, H. Zhao, B. Parashar, B. M. Sood, P. S. Mahadevia, H. P. Klinger, B. Vikram, **P. Mohan R. Achary**. 2003. Microsatellite dinucleotide (T-G) repeat: A

candidate marker for breast metastasis. **Cancer Detection and Prevention** 27: 19-23. (Annexure II)

Hui Zhao, Meena Jhanwar-Uniyal, Prasun K. Datta, Srishailam Yemul, Lap Ho, Gregory Khitrov, Ilya Kupershmidt, Giulio M. Pasinetti, Raghbir S. Athwal and **P. Mohan R. Achary.** 2003, Expression Profile of Genes Associated with Antimetastatic Gene, nm23-Mediated Metastasis Inhibition in Breast Carcinoma Cells. **International Journal of Cancer** (revised version submitted on 6-19-03). (Annexure III)

R. Yuan, S Fan, **P. Mohan R. Achary,** D. M. Stewart, I. D. Goldberg, and E.M.R. Rosen, (2001) Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/scatter factor (HGF/SF) in the setting of DNA damage. **Cancer Research** 61: 8022-8031. (Annexure IV)

C. Xue, H. Zhao, **P. Mohan R. Achary** and J. Segall. cDNA expression profile in EGFR and ErbB2 mediated metastasis in human breast carcinoma cell lines and their effect on spontaneous metastasis in a SCID mouse model. **Cancer Cell.** (Manuscripts in Preparation).

Directly not related to the grant (US Army IDEA grant sponsorship to the PI is acknowledged):

P. Mohan R. Achary, W. Jaggernauth, E. Gross, A. Alfieri, H. P. Klinger and B. Vikram (2000). Cell lines from the same cervical carcinoma but with different radiosensitivities exhibit different cDNA microarray patterns of gene expression. **Cytogenetics and Cell Genetics 91: 39-43.** (Annexure V)

L. H. Herbst, R. Chakrabarty, P. A. Klein, and **P. Mohan R. Achary,** (2001). Differential Gene Expression Associated with Tumorigenicity of Cultured Green Turtle FP-Derived Fibroblasts. **Cancer Genetics and Cytogenetics** 129: 35-39. (Annexure VI)

B. M. Sood, **P. Mohan R. Achary**, M. Fazzari and B. Vikram. 2003. The predictive value of gross residual disease after high-dose rate brachytherapy in locally advanced carcinoma of the cervix. Cancer. (Submitted)

Abstracts and Presentations: (Annexure VII)

Related to the grant:

P. Mohan R. Achary, Molecular markers of breast and cervical carcinomas, **Faculty meeting of the Department of Radiation Oncology, AECOM/MMC** on January 4th, 1999.

P. Mohan R. Achary, B. Mukherjee, Z. Fan, P. Mahadevia and B. Vikram (1999). Search for novel molecular markers to identify patients with breast cancer at high risk for developing metastasis using Representational difference analysis. **90th Annual AACR**

meeting, Philadelphia. **Proceedings of the American Association for Cancer Research,** April 10-14, 1999,431.

P. Mohan R. Achary, Molecular markers of breast metastasis and cervical carcinomas, **Faculty meeting of the Department of Radiation Oncology, AECOM/MMC** on January 3rd, 2000.

R. Khaimov, E. Gross, J. Liu, J. B. Vikram, and P. Mohan R. Achary,. Identification of gene expression profile in a highly metastatic breast carcinoma cell line by cDNA microarray method. **91st Annual AACR meeting,** April 1- 5, 2000, **Proceedings of the American Association for Cancer Research. 41: 431.**

P. Mohan R. Achary, R. Khaimov, Z. Fan and B. Vikram, Isolation of Molecular markers of metastasis in mammary carcinoma. **Proceedings of the 92nd Annual AACR meeting, New Orleans, LA**. March 28th-April 1st, 2001.

P. Mohan R. Achary, Molecular markers of breast metastasis and cervical carcinomas, **Faculty meeting of the Department of Radiation Oncology, AECOM/MMC** on January 6th, 2001.

Lilianette Rovira and P. Mohan R. Achary, To identify differential expression of genes associated with breast metastasis, **CUNY Conference in Science and Engineering, NY,** February 23rd, 2001.

P. Mohan R. Achary, Molecular markers of breast metastasis and cervical carcinomas, in the **Genetics Research Unit, Calcutta University, India** on May 15th, 2001.

Zhao H, Fan Z, Mukherjee B, Herbst L, Jones J, Klinger HP, Vikram B and P. Mohan R. Achary, Characterization of Metastasis Associated Gene Sequences in Breast Carcinoma. **Fifth Annual Einstein Postdoctoral Symposium, AECOM, December 4th, 2001.**

P. Mohan R. Achary, Metastasis associated genes in breast carcinoma, **Faculty meeting of the Department of Radiation Oncology, AECOM/MMC** on January 7th, 2002.

H. Zhao, Fan Z, Herbst L, Breining D, Jones JG, Mahadevia PS, Klinger HP, Vikram B, P. Mohan R. Achary. A candidate metastasis associated genetic marker for ductal mammary carcinoma. **93rd Annual AACR Meeting** at San Francisco, California, **Proceedings of American Association for Cancer Research,** April 6-10, 2002, (Abstract # 220).

External panelist and speaker in the **Human Genome Project conference** at Old Dominion University (Norfolk, VA) and presented a talk on breast metastasis, during June 14th and 15th 2002

Invited by the Department of Medical Oncology, Montefiore Medical Center to give a talk on the "Molecular markers in breast and cervical Cancer" on August 16th 2002.

Presentation (Molecular markers of metastasis in ductal mammary carcinoma) at the US Army 'Era of Hope' breast cancer research conference to be held during September 25th to 28th, 2002, in Orlando, Florida

H. Zhao, Jhanwar-Uniyal M, Anand V, Kuperschmidt I, Khitrov G, Herbst L and **P. Mohan R. Achary.** 2003, Genes and pathways associated with nm23 mediated metastasis inhibition in breast carcinoma cells. **94th Annual AACR Meeting** at Toronto, Canada, **Proceedings of American Association for Cancer Research,** April 4-7, 2003 (Abstract # 189).

Unrelated to the grant:

P. Mohan R. Achary, J. Wayne, H. P. Klinger and B. Vikram (1999). cDNA microarray gene expression analysis of cervical carcinoma cell lines with different degrees of radiosensitivity. **Proceedings of conference on "Prediction of Tumor Response to Therapy: Molecular Markers and the Microenvironment" at McGill University, Montreal, Canada,** October, 8-9, 1999, Pg. 9.

P. Mohan R. Achary, Gene expression markers in cervical carcinoma, Semi-annual meeting of the **Radiation Therapy Oncology Group, Philadelphia,** June 26th, 1999.

P. Mohan R. Achary, J. Wayne, H. P. Klinger and B. Vikram (2000). Gene expression analysis of cervical carcinoma cell lines with different degrees of radiosensitivity by cDNA microarrays. **Intl. Conference on Translational Research and Strategies in Clinical Radio-Oncology,** March 5-8th, 2000. **Switzerland. Int. J. Radiation Oncology Biol. Phys. 46:736.**

P. Mohan R. Achary, J. Wayne, E. Gross, R. Khaimov, H. P. Klinger and B. Vikram, Gene expression profiles in radioresistant cervix cancer cell lines by cDNA microarray. **91st Annual AACR meeting,** April 1- 5, 2000, San Franscisco. **Proceedings of the American Association for Cancer Research. 41: 709.**

P. Mohan R. Achary, Molecular markers of cervical tumor cell lines sensitive to radiation and cisplatin, **The 8th meeting of the New York Human Genetics Club, Memorial Sloan-Kettering Cancer Center,** October 14th, 2000.

L. H. Herbst, R. Chakrabarty, P. A. Klein and P. Mohan R. Achary, Differential Gene Expression Associated with Tumorigenicity of Cultured Green Turtle Fibropapilloma-Derived Fibroblasts. 92nd Annual AACR meeting, **New Orleans, LA.** Proceedings of the American Association for Cancer Research, **March 24-28th, 2001,**

Meena Jhanwar-Uniyal, Gina Day Stephenson, Renee Royak-Schaler, Chung-Xiou Wang, **P. Mohan R. Achary**, Anthony P. Albino and John Whysnor L. 2003, Involvement of p53 and BRCA1 genes in breast cancer in African-American and white women. **94th Annual AACR Meeting** at Toronto, Canada, **Proceedings of American Association for Cancer Research,** April 4-7, 2003 (Abstract # 189)

Patents and licenses applied for and/or issued

None

Degrees obtained that are supported by this award

None

Development of cell lines, tissue or serum repositories (core facilities)

1. Several breast carcinoma cell lines were obtained from ATCC and other scientists. Some tumors and matched positive lymph node material were obtained from Co-Operative Human Tissue Network and from the Pathology Department of Albert Einstein College of Medicine and Montefiore Medical Center.

2. We have purchased a LCM equipment and established a core LCM facility at the Department of Surgical Pathology of MMC/AECOM.

Informatics such as databases and animal models etc.

A tumorigenicity cum metastasis mouse model has been established to evaluate breast carcinoma cell lines transfected with the candidate metastasis associated gene sequences and also to evaluate the efficacy of potential anticancer drugs. In our mouse model, we inject human mammary carcinoma cell lines (MDA-MB-435) into mammary fat pads of the nude mice and after 6 weeks the primary tumors are surgically removed and after about 6-8 weeks the animals are killed to measure incidence of metastasis in lungs. We used this model to study signal transduction pathways associated with nm23/ErbB2 mediated breast metastasis inhibition/promotion.

Research protocols approved at AECOM:

1. Principal Investigator: Isolation of novel genetic lesions in cancer patients by representational difference analysis of archival tissues (i. Molecular markers of metastasis in ductal mammary carcinoma; ii. Markers for sensitivity of cervical cancers to therapies), AECOM CCI# 96-119.

2. Principal Investigator: An In Vivo model to study metastasis in breast cancer, AECOM AIC protocol# 000611.

3. Co-Principal Investigator (P.I: Dr. Joseph A. Sparano): Molecular Basis for Metastasis and Resistance to Hormonal Therapy in Early Stage Breast Cancer: ER-alpha Mutations, Caveolin-2 Mutations, and Metastasis Associated Genes.

Funding applied for based on work supported by this award

Research Projects and grant Support related to the Idea project:

1. **Principal Investigator**: Applied and awarded a fellowship by I. H. P. Klinger Fund (AECOM) for three years to hire a post-doc. to work on breast and cervical cancer research projects in P. I's lab. Duration: 2001-2003. Dr. Hui Zhao worked in PI's lab as a post doc sponsored by the foundation from 2000-2003 **(Funded).** (Annexure VIII)

2. Principal Investigator (50%): Markers of Metastasis in Ductal Mammary Carcinoma. Agency: **NIH**; Type: **R-21 (1 R21 CA97208-01),** Priority Score: **243. (Not funded)**

3. Principal Investigator (20%): Diagnostic Gene Expression Profiles in Breast Cancer. Agency: **NIH**; Type: **R-21 (PAR-99-128)** Number: **1 R21 CA87347-01**; Priority Score: **238**. **(Not funded)**

4. **Principal Investigator** (40%): "Identification and characterization of novel metastasis associated genes in Ductal Mammary Carcinoma"; Agency: **US Army Breast Cancer Research Program**; Type: Idea grant; Duration: 2004-06 **(Under Review)** (Annexure IX)

Research Projects and grant Support <u>unrelated</u> to the Idea project:

1. **Co-investigator** (10%):"The viral pathogenesis of marine turtle fibropapillomatosis" (P.I: Dr. Lawrence Herbst, AECOM); Agency: **Morris Animal Foundation**; Duration: 2003- 2006. **(Funded)** (Annexure X)

2. **Co-investigator** (10%):"Biochemical cytology of normal and malignant liver tissues" (P.I: Dr. Phyllis Novikoff, AECOM); Agency: **NCI/NIH**; Type: **R-01**; Duration: 2000- 2004. **(Funded)**

3.**Co-Principle Investigator** (20%): "Can the Patterns of Gene Expression by cDNA Microarray Predict the Clinical Outcome in Cervix Cancer: A correlative Study with RTOG 1990-2001" (P.I: B. Vikram, AECOM/MMC). Agency: Radiation Therapy Oncology Group; Duration: 1999-2001. **(Funded)**

4.**Consultant** (5%):"Characterization and mechanisms of radiation induced kidney injury" (P.I: Dr. Elias Lianos, Univ. of Med. and Dentistry of New Jersey); Agency: NIH; Type: R-01; Duration: 1997-2001. **(Funded)**

Employment or research opportunities applied for/or received on experiences/training supported by this award

1. The PI was an Instructor in the Department of Radiation Oncology at the time the grant was submitted. He has been promoted to an **Assistant Professor** on receipt of the **US Army Idea grant award**.

2. Dr. Achary has been elected as Editor for the journal, **Cytogenetic and Genome Research** (Annexure XI)

3. He has been selected as reviewer for journals namely, **Gynecologic Oncology, American Journal of Pathology, Cancer Detection and Prevention, Clinical Cancer Research, and Applied Immunohistochemistry and Molecular Morphology**.

4. He has been offered the position of 'Associate Administrator for Scientific Affairs' for the **International Cytogenetics and Genome Society**. (Annexure XII)

5. He has been identified as reviewer for the grant applications from the **Foundation of Ohio Cancer Research Associates**.

6. He has been selected as member in different scientific organizations namely, **American Association for Cancer Research** (Active); **Radiation Therapy Oncology Group** (Active); **American Society for Therapeutic Radiology and Oncology** (Associate); **RTOG cervical tumor tissue committee** (Member); **American Association for the Advancement of Science** (Active) and **International Cytogenetics and Genome Society** (Life).

7. He has been offered with a faculty position at the Fels Institute for Cancer Research, Temple University in 2002, which he accepted in March 2003.

8. Taught and supervised **medical residency fellows, post doctoral fellows, graduate students** of Radiation Oncology of MMC/AECOM **and summer students** from different Institutions. (Note: The expenses incurred by these research projects were funded by their respective Institutions).

i. Post doctoral and medical residence fellows (Supervised):
Purnima Banerjee, Ph.D.
Zuoheng Fan, MD
Rafik Khimov, MD
Bhupesh Parashar, MD
Weinright Jaggernauth, MD
Udayan Guha MD, PhD
Jaijan Liu, PhD
Yemul Srishailam, PhD
Hui Zhao, PhD

ii. Graduate students (Supervised):
Adel Nour PhD student, Yeshiva University, NY.

iii. Summer Students (Supervised)
Avi Holchendler, Yeshiva University, NY
Emmanuel Olubiyi, Bronx Community College, NY
Shlomo Koyfman, Yeshiva University, NY
Isaiah Friedman, Yeshiva University, NY
Seven Edward Quatela, MD/PhD student, NYU, NY
Olayemi M Ikusika, Johns Hopkins University, MD
Azizirad Omeed, Yeshiva University, NY
Stephanie Rice, St. John's University, NY
Lilianette Rovira, Bronx Community College, NY
Maya Kolipakam, Columbia University, NY
Shane Rabin, Yeshiva University, NY
Eugene J. Gross, Yeshiva University, NY
Rafael Abramov, Yeshiva University, NY
David Day, The Bronx High School of Science, NY
Steven Martinez, Bronx Community College, NY
Shruti Tewari, Temple University, PA

(8) Conclusions:

Breast metastasis disease is a complex process involving multiple steps and therefore global genetic profiling studies of metastatic (13%; group I) and non metastatic (87%; Group-II) tumors are required to elucidate this process. In order to isolate markers for breast metasatsis, we have used several powerful technical resources to isolate molecular genetic markers specific to breast metastasis. Archival tissue samples were used to retrospectively correlate the molecular markers with the clinical outcome of patients. Pure populations of tumor cells without contamination of stromal cells were isolated by single cell and laser capture microdissection methods. Representational difference analysis (RDA) method was used to isolate metastasis-specific genetic markers by comparing normal, primary and metastatic cell populations. Fifteen candidate MADS have been isolated from 12 RDA experiments. Three of them (MADS-IV, IX and XI) were found to be promising markers for breast metastasis. RH mapping and homology search results indicated that MADS-IX is present close to PTEN gene on 10 q chromosomal arm. Screening of MADS-IX on different tumor samples and tumor cell lines (one of them with homozygous loss of PTEN gene) revealed that MADS-IX is neither a part of PTEN gene nor localized in the homozygous loss region of chromosome 10q arm encompassing PTEN. Our attempts to supplement the PCR method with Fluorescence in situ hybridization (FISH) for screening MADS on primary tumor tissue touch preparation cells were successful. The results indicate the potential of the MADS-IX as a FISH probe in distinguishing the primary tumors that did and that did not develop metastasis. A tumorigenesis cum metastasis SCID mouse model has been developed and was successfully used to evaluate the metastatic potential of MDA-MB-435 breast carcinoma cells transfected with a metastasis suppressor gene (nm23) and a metastasis promoter gene (ErbB2). After the evaluation of the MDA-MB-435 cells transfected with nm23 and ErbB2 genes, they were subjected to cDNA microarray studies which resulted in the discovery of several known/novel genes associated with nm23 mediated metastasis inhibition and ErbB2 mediated metastasis promotion and identification of drug targets.

(9) References:

Achary et al. Breast Cancer Research 5: 52-58 (2003)
Emmert-Buck et al. Science 274:998-1001 (1996)
Li et al. Science 275: 1943-1947 (1997)
Lisitsyn et al. Science 259: 964-951 (1993)
Mejia and Monaco. Genome Research 7: 179-186 (1997)
Mukherjee et al Cancer Detection and Prevention 27: 19-23 (2003)

APPENDIX COVER SHEET

ATTACHMENTS I - XII

Breast Cancer Research Vol 5 No 2 Achary *et al.*

Research article

Open Access

A candidate metastasis-associated DNA marker for ductal mammary carcinoma

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[3]Department of Pathology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, New York, USA
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Abstract

Background: Molecular genetic markers to identify the 13% lymph node-negative mammary carcinomas that are prone to develop metastases would clearly be of considerable value in indicating those cases in need of early aggressive therapy.

Methods: Representational difference analysis was used in an attempt to identify genetic alterations related to breast cancer metastasis by comparing genomic DNA from microdissected normal cells and from metastatic cells of ductal breast carcinoma patients.

Results: Representational difference analysis products yielded 10 unique metastasis-associated DNA sequences (MADS), i.e. products apparently lost in metastatic cell DNA. Of these sequences, MADS-IX was found to be lost in the transition from primary to metastasis in two out of five ductal breast carcinoma cases. This sequence was localized on chromosome 10q21 by radiation hybrid mapping and fluorescence *in situ* hybridization. The *PTEN* gene, which is also located on chromosome 10q, was detected to be present by PCR in all five cases. On the contrary, a breast carcinoma cell line, HCC-1937, which has homozygous loss of a region encompassing the *PTEN* gene, showed the presence of MADS-IX. PCR screening of three additional breast carcinoma cell lines with known losses in specific chromosomal regions also showed the presence of MADS-IX.

Conclusion: These data suggest that MADS-IX possibly is part of a novel candidate metastasis-associated gene located close to the *PTEN* gene on chromosome 10q. The first set of PCR screening in five patient samples indicates that it could be used as a molecular marker for ductal mammary metastasis.

Keywords: archival tumor samples, mammary carcinoma, metastasis, molecular markers, representational difference analysis

Introduction

The clinical outcome is generally positive for patients with node-negative breast carcinoma (i.e. those patients without detectable metastases in the lymph nodes) who have been treated with surgery or with surgery and radiation therapy. The disease spreads in about 13% of the patients, however, and their lives are at risk of death [1–3]. Genetic tests capable of identifying patients at risk for metastatic spread and/or better treatment targeted to eradicate metastatic tumor deposits could have a dramatic impact on the overall survival of these patients.

The construction of a panel of molecular genetic markers for identifying the 13% lymph node-negative mammary carcinomas that are prone to develop metastases [3] would be valuable in indicating those cases in need of early

bp = base pairs; FISH = fluorescence *in situ* hybridization; LCM = laser capture microdissection; MADS = metastasis-associated DNA sequences; NCBI = National Center for Biotechnology Information; PCR = polymerase chain reaction; RDA = representational difference analysis; SCM = single cell microdissection; STS = sequence tagged sites.

aggressive therapy. There would also be considerable benefit to the 87% of women with mammary carcinomas who are not likely to metastasize by sparing them the physical, mental and financial costs of the treatment. In addition, further knowledge of the genetic mechanisms that play an important role in metastasis could ultimately lead to the development of improved therapeutic procedures.

The evidence for the role of gene alterations in promoting metastasis in general, and in mammary carcinomas in particular, is still accumulating. It is probable, however, that like tumorigenicity suppressor genes and oncogenes many more genes involved in metastasis remain to be discovered since the process is complex, involving a large number of pathways [4]. Most of the known genetic mechanisms involved in the progression of a tumor to the metastatic state involve the loss of function of genes that prevent cells from becoming invasive. These are similar to tumorigenicity suppressor genes, whose loss of wild-type growth regulatory function leads to unregulated or malignant growth. Similarly, the wild-type metastasis suppressor genes regulate the cell's mobility and its response to environmental messages, presumably keeping most cells localized and differentiated. Mutation or complete loss (deletion) of the wild-type counterpart of any such gene in a malignant cell may make that cell more motile, invasive or metastatic. In particular, mutations of genes regulating cell adhesion molecules have been reported to be metastasis-fostering alterations [5–8]. In human breast cancers, several other genes have been found to be associated with metastasis, namely *nm23* [9,10], *KAI1* [11,12], *mta1* [13], *KiSS1* [14], *PTEN/MMAC1* [15,16] and *BRMS1* [17].

Representational difference analysis (RDA), a DNA subtractive hybridization method [18], was used in the present study to test the working hypothesis that, to achieve the metastatic state, primary mammary carcinoma cells must acquire genetic changes in addition to those changes that led to transformation. With the RDA method, DNA from normal cells was compared with that of the metastatic cells of the same patient. The recovered differential sequences were then mapped on human chromosomes and used to screen DNA samples from normal, primary and metastatic cells of five additional ductal mammary carcinoma patients to determine whether these sequences were consistently associated with metastasis.

Materials and methods

Tissue samples and cell lines

Normal breast tissue and lymph nodes with metastatic cells of a 52-year-old patient (C-1050) with ductal mammary carcinoma were provided by the Cooperative Human Tissue Network. Additional matched normal, primary and metastatic tissue samples were collected from four patients (patient 1, C-18805; patient 2, C-98-05H; patient 3, C-19898; and patient 4, C-20635) from the Cooperative Human Tissue Network and from one patient (patient 5, DS-9605) from the Surgical Pathology Department of Albert Einstein College of Medicine and Montefiore Medical Center. The DNA samples from mammary carcinoma cell lines (HCC-1806, HCC-11433, HCC-1428 and HCC-1937) and matched normal cell lines were received from Dr R Parson of Columbia University, New York, USA.

Isolation of cells from biopsy samples by single cell microdissection

Single cell microdissection (SCM) was performed on hematoxylin and eosin-stained tissue sections of positive lymph nodes from the ductal breast cancer patient samples [19]. In the present method of SCM, a Zeiss axiovert phase contrast photomicroscope fitted with a television monitor and a Narashige mechanical microdissector was used. A glass micropipette was used to draw up individual cells after dissection.

Tumor cells were identified in the microscope and, using the tip of a glass micropipette attached to a syringe, they were dissected out without disturbing the surrounding tissue and were then drawn into the pipette. After five to 10 cells were collected in the tip, it was broken off and dropped into a sterile eppendorf tube. Approximately 10,000 metastatic tumor cells were collected in this manner for RDA experiments. The SCM method was used to isolate tumor cells for the RDA experiment because laser-capture microdissection (LCM) equipment was initially not available.

Isolation of cells from biopsy samples by LCM

LCM [20] was used for isolating tumor cells from primary and metastatic tissue samples of additional patients for screening candidate metastasis-associated DNA sequences (MADS) by PCR. After many tumor cells have been captured on the LCM cap, it is then placed on a 500 µl PCR tube containing ATL lysis buffer (Qiagen Inc, Valencia, CA, USA) and the DNA is extracted.

DNA extraction

About 1 µg DNA was isolated from approximately 10,000 cells microdissected from metastatic tumor and normal (glandular and stromal) breast tissue samples, using a modified method of DNA extraction from archival tissues. Briefly, the microdissected cell pellet was incubated overnight in lysis buffer (Qiagen Inc) with proteinase-K at 55°C. Glycogen (carrier) was added to the cell lysate and DNA was extracted using a phenol–chloroform–isoamyl alcohol mixture followed by ethanol precipitation [19].

RDA and characterization of differential products

The RDA procedure was basically as described by Lisitsyn and coworkers [18,21] using the *BglII* representation. The metastatic cancer cell DNA was used as the 'driver'

and the DNA from the normal cells was the 'tester'. Three rounds of hybridization were performed to subtract the common sequences. The primers used for representation and hybridization steps of RDA are presented in Table 1. DNA from the differential bands (lost in metastasis) was cloned using the TA cloning system (pCR2.1; Invitrogen Co, Carlsbad, CA, USA). Clones were selected at random and used as α^{32}P-dCTP (Amersham Biosciences Corp, Piscataway, NY, USA) probes on dot blots or Southern blots containing amplicon DNA from the original normal and metastatic cells to verify whether these sequences are actually present in the normal cell DNA sample and missing in the metastatic cell DNA samples. Positive clones (showing signals in normal cell DNA and missing in metastatic cell DNA) were sequenced. A homology search was performed using the National Center for Biotechnology Information, National Institutes for Health, USA (NCBI) Blast program and physical mapping.

Radiation hybrid mapping

Radiation hybrid mapping was used to localize the MADS on human chromosomes using high-resolution GeneBridge 4 radiation hybrid panels (Research Genetics, Inc , Carlsbad, CA, USA). Based on the sequences of differential products isolated from RDA experiments, 10 pairs of PCR primers were designed for 10 groups of candidate gene sequences. Based on the PCR results, chromosome localization of these sequences was determined using the Whitehead Institute/MIT databases [22].

Fluorescence *in situ* hybridization of MADS-IX

Chromosome preparations were made from phytohemagglutinin-stimulated peripheral blood lymphocyte cultures from normal individuals. The slides were fixed in methanol–acetic acid and were air-dried. The probes used for fluorescence *in situ* hybridization (FISH) were the Spectrum Green labeled centromere of 8 (Vysis, Downers Grove, IL, USA) and the Spectrum Green labeled 2 kb sized MADS-IX sequence. Primers were designed from the BAC clone (NCBI, NIH, USA, RP11-407) containing the MADS-IX sequence to obtain a 2 kb sized PCR product encompassing the MADS-IX region (Table 1).

The 2 kb DNA fragment was run on a 1% gel, isolated from gel and eluted in water, and was labeled with Spectrum Green using the nick translation method, following the manufacturer's instructions (Vysis). Three-day-old slides were denatured in 70% formamide/2 × SSC (sodium citrate/chloride) solution at 74°C. The dehydrated and air-dried slides were used for hybridization according to published methods with some modifications [23,24]. The slides were counter-stained with 4,6-diamidino-2-phenylindole dihydrochloride (Molecular Probes, Eugene, OR, USA) and images were captured on the Nikon E800 using the Quips Pathvysion system (Applied Imaging, Santa Clara, CA, USA).

Results

Isolation of candidate MADS

RDA was performed with the DNA recovered by SCM from normal cells and from metastatic cells in the lymph nodes of a patient with ductal mammary carcinoma. Since the objective was to isolate sequences that are lost in the progression of cells from normal to metastasis, we focused on the products of RDA in which the metastatic cell DNA was used as the driver and the normal cell DNA was the tester.

As shown in Fig. 1, RDA differential products were isolated and DNA was extracted from each of the five bands obtained from the loss side of the third round of hybridization, and DNA from each band was cloned separately. One hundred clones from each DNA band were saved and a subset of 100 clones (20 clones from each differential product) was selected randomly for further characterization. These 100 clones were probed (labeled with α^{32}P-dCTP) on dot blots containing normal cell DNA and metastatic cell DNA. It was found that 79 clones hybridized only with the normal cell DNA and did not hybridize with DNA from the metastatic cells (Fig. 2a). The remaining clones hybridized with both normal cell DNA and the metastatic cell DNA. This may have been due to incomplete subtraction. Of the 79 clones that hybridized only with the DNA of the normal cells, 50 were selected and tested further by Southern blotting to verify the RDA results. None of these clones hybridized with the DNA of the metastatic cells, and all were detected in normal cells (Fig. 2b).

Sequencing and homology search of candidate MADS

RDA derived 50 clones that were sequenced and those with identical sequences were grouped. Ten unique candidate MADS were thus identified. The sequence homologies found by searching the nucleotide databases of these MADS are presented in Table 2. The search revealed that these MADS have homology with sequences on chromosome 1 (MADS-III), chromosome 5 (MADS-I), chromosome 6 (MADS-VII and MADS-VIII), chromosome 7 (MADS-VI), chromosome 10 (MADS-IX), chromosome 15 (MADS-X), chromosome 16 (MADS-V) and chromosome 21 (MADS-II). Nine of these MADS have about 82–99% homology to known human DNA sequences, and the remaining MADS-IV was found to be novel, having no homology with the sequences in the gene banks.

Radiation hybrid mapping of MADS

We used the GeneBridge 4 Radiation Hybrid panel (Research Genetics, Inc) to localize these 10 MADS on human chromosomes. For this physical mapping, and also to be able to use these sequences to screen additional patient samples, primer pairs were designed for each sequence and tested by PCR on a positive control (human genomic DNA) and on two negative controls (Chinese hamster genomic DNA and no template). Of the 10 MADS

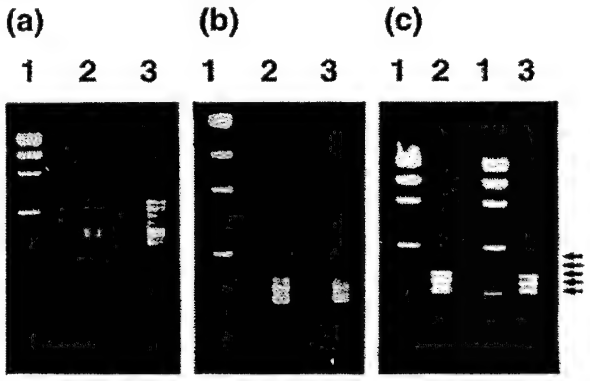
Table 1

Primers used in representational difference analysis (RDA) and different PCR screening experiments

Primer/product size	Primer sequence (forward)	Primer sequence (reverse)
RDA representation	R24, 5′-agcactctccagcctctcaccgca-3′	R12, 5′-gatctgcggtga-3′
RDA first-round hybridization	J24, 5′-accgacgtcgactatccatgaaca-3′	J12, 5′-gatctgttcatg-3′
RDA second-round hybridization	N24, 5′-aggcaactgtgctatccgagggaa-3′	N12, 5′-gatcttccctcg-3′
RDA third-round hybridization	Same as first round (J24)	Same as first round (J12)
PTEN (556 bp)	5′-ctcagattgccttataatagtc-3′	5′-tcatgttactgctacgtaaac-3′
MADS-IX (180 bp)	5′-aggtaggtagagtaacaggtttgttt-3′	5′-gatctgttctccctttttctttagctt-3′
FISH fragment (2 kb)	5′-actatgtatgtgagcatctgtttgc-3′	5′-tgcactcttcacttgagtaaacttg-3′
β-Actin (295 bp)	5′-tcacccacactgtgcccatctacga-3′	5′-cgacgtagcacagcttctcctta-3′

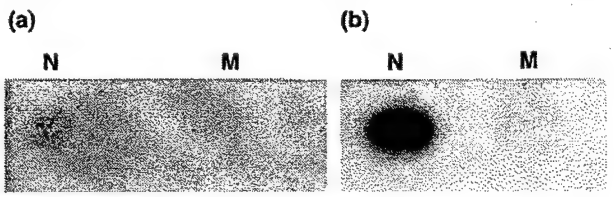
FISH, fluorescence *in situ* hybridization.

Figure 1



Isolation of representational difference analysis (RDA) differential products from single-cell microdissected archival breast tissues. RDA hybridization was of the normal cell DNA versus the metastatic tumor cell DNA and was performed in two ways. In the first RDA (lane 2) the metastatic cell DNA was used as the tester (which should yield differential sequences gained during malignant transformation or in the process of becoming metastatic), and in the second RDA (lane 3) normal DNA was used as the tester (which should yield sequences that were lost from the metastatic cells). **(a)**, **(b)** and **(c)** Three rounds of RDA hybridizations were performed. In the third round of hybridization, the 'gain' lane contained four DNA bands (faint fourth band) ranging from 200 to 300 bp in size (c, lane 2) whereas in the 'loss' lane there are five bands (faint fourth band) ranging from 200 to 370 bp (c, lane 3) in size (arrows). Lane 1, low molecular weight DNA marker (2 kb).

Figure 2



Southern blot analysis using MADS-IX as an α^{32}P-dCTP probe on normal amplicon DNA and on metastatic amplicon DNA. **(a)** A dot blot and **(b)** a Southern blot showing positive signals only in the normal cell DNA (N) but not in the metastatic cell DNA (M).

tested, only five (MADS-IV, MADS-VII, MADS-VIII, MADS-IX and MADS-X) were successfully mapped to specific human chromosomes (Table 2). Of these five MADS, MADS-IV did not show any match with gene bank sequences and therefore appeared to be a novel DNA sequence. The chromosome localizations for MADS-VII, MADS-VIII, MADS-IX and MADS-X obtained by physical mapping and by homology search data were in agreement.

Further characterization of MADS-IX

While the homology results of MADS-IX revealed that it has 94% homology with the sequence on chromosome 10q (BAC-AC022541.10), the radiation hybrid mapping showed that MADS-IX is localized to a 21cR interval between markers D10S539 and D10S549, corresponding to human chromosome 10 band q21.1 [25]. To confirm that MADS-IX is not an artifact and is actually a human DNA sequence located on chromosome 10q, we used the sequence as a fluorescence *in situ* hybridization probe on human metaphase chromosomes. Based on the sequence of the BAC clone (NCBI, RP11-407) encompassing MADS-IX, a 2 kb DNA fragment containing MADS-IX was generated by PCR. To localize MADS-IX cytologically, we labeled the 2 kb sized MADS-IX with Spectrum Green (Vysis) and hybridized on normal human metaphase chromosomes. Figure 3 shows that the MADS-IX probe was hybridized on the 10q21 region. The Spectrum Green labeled centromere of chromosome 8 (Vysis) was used as a control.

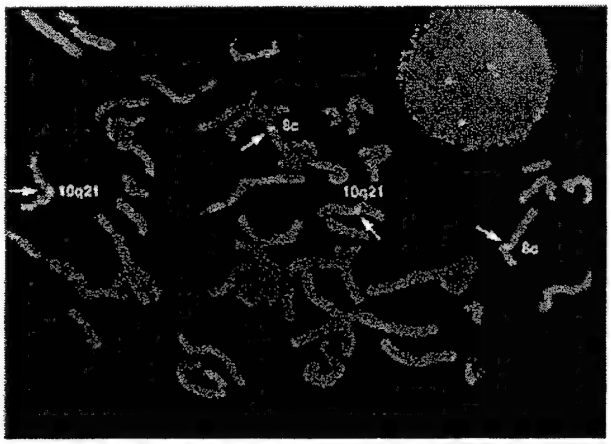
To determine whether MADS-IX is indeed associated with metastasis, DNA was screened from cells recovered with the LCM method from normal, primary and metastatic tissue

Table 2

The length, homology search and radiation hybrid mapping results of the metastasis-associated DNA sequences (MADS)

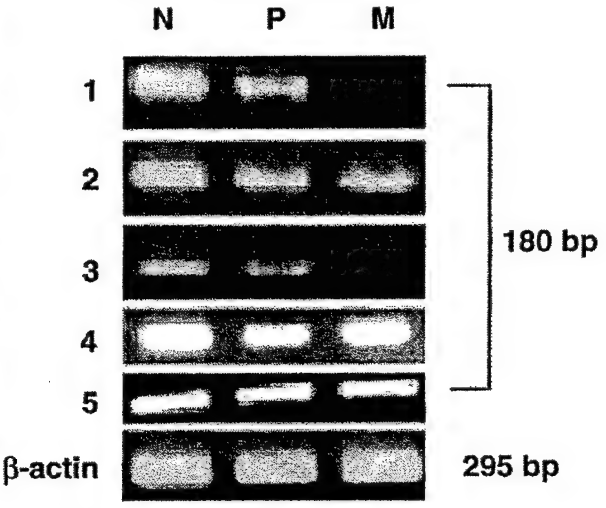
MADS	Length (bp)	Homology	Radiation hybrid mapping
MADS-I	205	Chromosome 5 (97%) AC005915.1	Not successful
MADS-II	144	Chromosome 21 (99%) AC010463.6	Not successful
MADS-III	277	Chromosome 1 (97%) AL359265.8	Not successful
MADS-IV	185	No match	Chromosome 5; places 19.72 cR from WI-6737
MADS-V	220	Chromosome 16 (98%) AC004234.1	Not successful
MADS-VI	242	Chromosome 7 (97%) AC09333.1	Not successful
MADS-VII	190	Chromosome 6 (98%) AL121935.17	Chromosome 6; places 1.51 cR from WI-3110
MADS-VIII	170	Chromosome 6 (82%) AC013429.12	Chromosome 6; places 3.67 cR from AFMA191WD1
MADS-IX	180	Chromosome 10 (94%) AC022541.10	Chromosome 10; places 5.66 cR from D10S546
MADS-X	192	Chromosome 15 (98%) AC104260.5	Chromosome 15; places 1.71 cR from D15S157

Figure 3



Localization of the 2 kb DNA fragment containing MADS-IX on human metaphase chromosomes. The centromere of chromosome 8 was used as a positive control probe. MADS-IX was found to localize around the 10q21 chromosome region (close to the centromere). Both the MADS-IX and the Spectrum Green labeled centromere of 8 were labeled with spectrum green (Vysis, Downers Grove, IL, USA), and the chromosomes were counter-stained by 4,6-diamidino-2-phenylindole dihydrochloride.

Figure 4

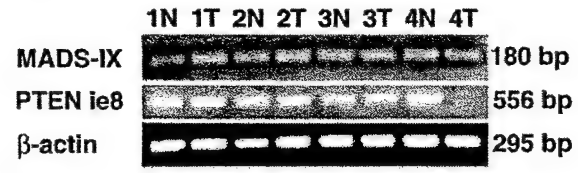


PCR screening of MADS-IX on normal cell DNA, primary cell DNA and metastatic cell DNA samples of five patients. PCR results show the target DNA band (180 bp) missing in patients 1 and 3. While MADS-IX showed a reduction in the intensity (about one-half) of the PCR band (heterozygous loss) in the primary tumors (P) of patients 1 and 3, it is completely missing (homozygous loss) in their metastatic (M) cell DNA samples but present in normal cell DNA (N). β-Actin was used as an internal control.

samples from five additional patients. Because MADS-IX was mapped to chromosome 10q21, we also wanted to determine whether it was located in the region encompassing a well-known metastasis-associated gene, *PTEN* [15].

First, DNA samples from these five patients were screened by PCR using primers designed for MADS-IX and *PTEN*. MADS-IX showed PCR amplification in normal cell DNA samples of all the five patients, but the intensities of the PCR bands were less in the primary tumor cell DNA samples and were totally undetectable in the metastatic cell DNA of patients 1 and 3 (Fig. 4). The *PTEN* gene, on the contrary, was detected, without any difference in the intensity of PCR bands in the DNA samples of normal

Figure 5



PCR screening of MADS-IX and *PTEN* on DNA samples from four tumor cell lines and matched normal DNA. Results (first row) show that MADS-IX is present in all the tumor cell lines (HCC-1806, HCC-1143, HCC-1428 and HCC-1937) especially the HCC-1937 cell line, which has loss of a region encompassing the *PTEN* gene. Screening of *PTEN* in these four cell lines (second row) show its presence in the HCC-1806, HCC-1143 and HCC-1428 tumor cell lines but, as expected, it is missing in HCC-1937. β-Actin was used as an internal control.

cells, primary tumor cells and metastatic lymph node cells of all five patients.

Second, MADS-IX and *PTEN* were screened in four breast tumor cell lines (HCC-1806, HCC-1143, HCC-1428 and HCC-1937) and matched normal DNA. The first three tumor cell lines had known losses in specific chromosomal regions, and the fourth had loss of homozygosity of the *PTEN* gene. PCR screening of MADS-IX and *PTEN* showed that MADS-IX is present in all four tumor cell lines, but *PTEN* is only present in cell lines HCC-1806, HCC-1143 and HCC-1428 and is missing in HCC-1937 (Fig. 5).

Discussion

Reports show a statistically significant increase in loss of heterozygosity events involving unknown genes on chromosome 16q [26], on chromosome 10q [15] and on chromosome 14q [27], in metastatic breast cancers. Measuring loss of heterozygosity, O'Connell *et al.* [27] found that the majority of lymph node-negative primary breast tumors did not amplify a region linked to D14S62 and D14S51, while lymph node-positive breast tumors retained heterozygosity for these markers. These data could imply the existence of metastasis promoting gene(s) in that region or, alternatively, the observed molecular changes may be used as a marker of metastatic propensity [28].

In the present investigation we used RDA in an attempt to identify genetic alterations related to breast cancer metastasis by comparing normal cell DNA and metastatic cell DNA of a ductal breast carcinoma patient. Ten unique candidate MADS were identified and the sequence homologies found by searching the gene bank of these MADS are presented in Table 2. The search revealed 82–99% homology to known human gene sequences for nine of these MADS but MADS-IV was found to be novel, having no homology with the sequences in the gene banks. Further characterization of MADS-IX revealed interesting information on the genes and sequence tagged sites (STS) markers surrounding it. Within a range of 1.4 Mb of MADS-IX, we noticed two *CDC2* genes (cell division cycle 2 protein, isoform 1 and isoform 2) and two splice variants of the *ZWINT* gene (without known exact function). In the range of 821 kb, there are nine STS markers (RH26942, SHGC-58267, SHGC-79901, AFM336XD1, SHGC-103705, SHGC-3856, SHGC-84937, STSG72171 and SHGC-81245).

Physical and cytological mapping of MADS-IX confirmed that it is located at 10q21.1. Screening of MADS-IX on DNA samples from five more sets of normal, primary and matched metastatic tumor samples (Fig. 4) revealed that MADS-IX is lost in the transition from normal cells to primary cells to metastasis cells in two of five cases (three of six cases if the case used in the RDA assay is included). PCR screening of *PTEN* on these five patient samples and screening of MADS-IX and *PTEN* on a breast carcinoma cell line that had loss of homozygosity of the *PTEN* gene revealed that MADS-IX is neither a part of the *PTEN* gene or localized in the homozygous loss region of chromosome 10q encompassing the *PTEN* gene. These findings together strongly suggest that MADS-IX is a candidate marker for a novel metastasis suppressor gene. Additional archival cases are being screened to determine whether MADS-IX is a reliable marker for metastasis in ductal breast cancers.

Competing interests

None declared.

Acknowledgments

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Microsatellite dinucleotide (T-G) repeat: a candidate DNA marker for breast metastasis

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Abstract

A dinucleotide (T-G) repeat sequence was isolated by comparing DNA from metastatic lymph node and matched normal breast samples from a ductal mammary carcinoma patient using representational difference analysis (RDA) method. Our present study used this metastasis associated DNA sequence (MADS) as a diagnostic probe to screen five patient samples by slot blot method. A new approach to isolate single cells by microdissection, namely single cell microdissection (SCM) was developed to obtain homogeneous population of tumor cells (~1000) from matched primary tumors and corresponding positive lymph nodes of five patients. We isolated DNA from these homogeneous tumor cells and used for the RDA and DNA slot blot experiments. The screening of patient samples showed loss of this MADS in the transition from primary to metastasis in four out of five cases (80%) suggesting its possible role in breast metastasis.

Keywords: Dinucleotide repeat; Metastasis; Metastasis associated DNA sequence (MADS); Representational difference analysis (RDA)

1. Introduction

The dissemination of cancer cells from primary site is the main cause for relapse of disease in patients with solid tumors. The degree of spreading of tumor cells in the circulation involves multiple tumor–host interactions through complex genetic mechanisms. Alterations in the expression of both the oncogenes and tumor/metastasis suppressor genes are known to be involved in metastasis [1–3]. Mutation or complete loss (deletion) of the wild-type counterpart of any suppressor gene in a malignant cell may increase cell motility, invasion or metastasis. Metastasis is a complex process involving a cascade of biochemical and genetic events that regulate growth, vascularization, invasion, transport and survival in the circulation followed by adhesion, extravasation, and proliferation at the distant sites [3]. In human and mouse breast cancers only few genes associated with metastasis have been reported [4–7].

We used the representational difference analysis (RDA) method [8–10] in an attempt to identify candidate DNA marker sequences and/or genes involved in the progression of the disease from benign to invasive breast carcinoma stage to metastasis by comparing DNA samples from a population of microdissected tumor cells from positive lymph nodes and matched normal cells from the same patient. Southern blots showed that most of the sequences (87%) were present in normal cell DNA and partially or completely missing in the metastasis cell DNA samples [10]. We found that one of these metastasis associated DNA sequences (MADS-XI) is enriched with novel dinucleotide (T-G) repeats. Since microsatellite repeats are known to regulate the expression of certain genes associated with metastasis [11–13], we made an attempt to determine if this novel gene sequence, MADS-XI could be used to predict the primary tumors that are prone to develop metastasis. We therefore screened additional five patient samples by DNA slot blot method to determine if this MADS-XI is actually involved in metastasis to lymph nodes in breast carcinoma.

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2. Materials and methods

2.1. Tissue samples

Archival tumor tissue material from five patients with ductal mammary carcinoma (coded case numbers: 1, C-14153; 2, C-1050; 3, DS97-919; 4, C-14852; 5, DS-711) were obtained from Co-operative Human Tissue Network (1, 2 and 4) and from the Surgical Pathology Department of Montefiore Medical Center (3 and 5). Each case consisted of primary and metastatic lymph node tumor tissue samples and matched normal tissue from the same patient.

2.2. Isolation of cells from biopsy samples by single cell microdissection

Cells from tumor biopsy samples were isolated using an innovative microdissection approach namely single cell microdissection (SCM). Thus, homogeneous populations of tumor cells (~1000) were isolated from matched primary tumors and corresponding positive lymph nodes of patient samples. SCM was performed on hematoxylin and eosin stained tissue sections of 5 μm thickness from primary tumors and metastatic lymph nodes (fresh frozen and fixed in OCT) from ductal breast carcinoma patient samples. In our method of SCM, a Nikon inverted photomicroscope fitted with a Narashige mechanical microdissector was used (Fig. 1). Tumor cells were identified in the microscope and using the tip of a glass micropipette attached to a syringe, they were dissected out one after the other without disturbing the surrounding tissue and then drawn into the pipette (Fig. 2a and b). After 5–10 cells were collected in the tip, it was broken off and dropped into a sterile eppendorf tube. As shown in Fig. 2c and d, pure population of tumor cells was isolated from a positive lymph node tissue sample. Compared to laser capture microdissection methods, this is less expensive and provides high degree of homogeneity of cell population. SCM method also alleviates any possible damage to the DNA or RNA of the microdissected cells as UV or infra-red sources of laser beams are not used in this method. Since the collection of large number of homogeneous cells by this method is very tedious and time consuming, we have modified RDA protocol and standardized a genomic DNA amplification method to isolate larger amounts of DNA from relatively few cells (~1000) for routine DNA slot blot [14] preparations.

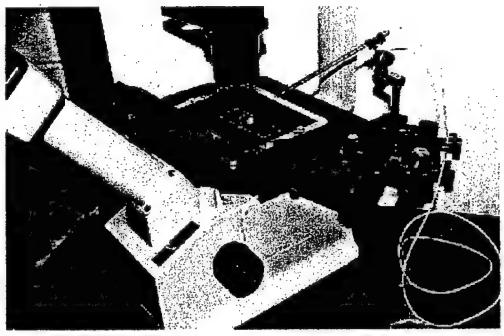

Fig. 1. Single cell microdissection apparatus. An inverted tissue culture Nikon photo microscope is attached with a Narashige micromanipulator for single cell microdissection. A capillary glass micropipette is fitted to the micromanipulator needle holder to dissect the tumor cells and a syringe is connected with a plastic tubing to the other end of the micropipette to draw tumor cells into its tip.

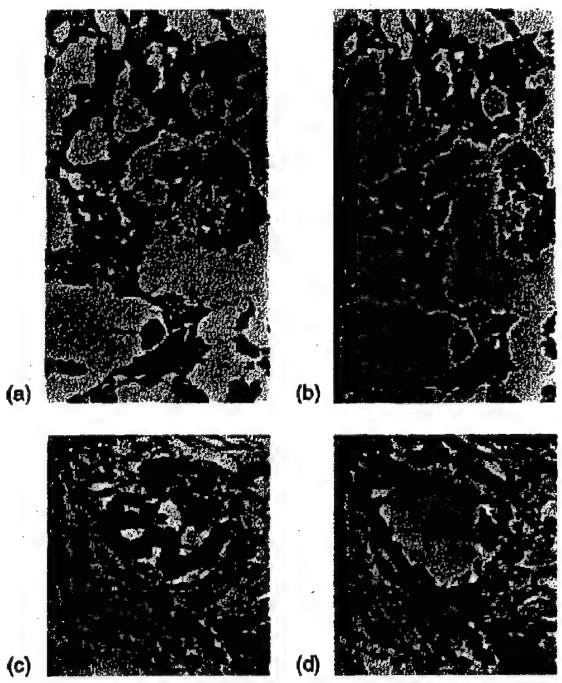

Fig. 2. Single cell microdissection of tumor cells. Dissection of one tumor cell from the primary tumor section of a ductal mammary carcinoma. A tumor cell (⇒) before (a) and after (b) microdissection. The arrows (→) are showing two reference cells and double arrow (⇔) showing intact collagen material (a) and the floating collagen (b) on the section. Tumor cells in a positive lymph node tissue (shown by thick arrow) before (c) and after (d) microdissection.

2.3. DNA extraction

About 100 ng of DNA was isolated from approximately 1000 cells microdissected from primary tumor and metastatic lymph node tissue samples, using modified methods of DNA isolation and genomic DNA amplification from limited cells. Briefly, the microdissected cell pellet was incubated with Proteinase-K (1 μl of 20 μg/ml stock) in a 20 μl of Tris-buffer for 1 h at 37 °C. The sample was denatured at 95 °C for 2 min, cooled to room temperature and *Bgl* II digestion (1:200 dilution of the stock 10 U/μl enzyme) was set up using 1 μl of the diluted enzyme in a 20 μl reaction mixture containing 100 ng of DNA. The digestion was carried for 2 h and the DNA fragments were ligated with the 24 mer and 12 mer oligos and *Bgl* II amplicons

were prepared [8,11]. PCR amplification of these *Bgl* II representation amplicons was performed using 24 mer oligo primers (95 °C 1 min, 72 °C 3 min for 30 cycles followed by 1 cycle at 72 °C for 10 min). DNA was extracted using phenol–chloroform–isoamyl alcohol mixture followed by EtOH precipitation. The DNA samples were quantified on an agarose gel using a high mass DNA maker (0.1–10 kb) and predetermined quantities of sheared salmon sperm DNA (0.1, 0.2, 0.5, 0.7 and 1 μg).

2.4. RDA and characterization of differential products

The RDA procedure was basically as described by Lisitsyn et al. [8,9] using *Bgl* II representation. The metastasis cancer cell DNA was used as the 'driver' and the DNA from the normal cells was the 'tester'. Three rounds of hybridization were performed to subtract the common sequences [8–10]. DNA from the differential bands (lost in metastasis) was cloned using the TA cloning system (pCR2.1; Invitrogen Co.). Clones were selected at random and used as ^{32}P-dCTP (Amersham) probes on dot/Southern blots containing DNA from the original normal and metastasis cells to verify whether these sequences are actually present in normal and missing in metastatic cell DNA samples [10]. Positive clones (showing signals in normal and missing in metastatic cell DNA) were sequenced and homology search performed using NCBI Blast program.

2.5. Slot blot method

The slot blot method was performed following published methods [14]. Briefly, the PCR amplified products were purified by phenol–chloroform method as mentioned elsewhere and suspended in 50 μl of TE buffer. One microgram of the quantified product was denatured with NaOH (1N) and incubated for 5 min at 37 °C. The final volume was made up to 100 μl with 10× SSC (final concentration of 6× SSC). The samples were applied on pretreated nitrocellulose membranes using Schleicher & Schuell (S&S) Minifold II slot-blotter apparatus following manufacturer's instructions. The membrane was UV cross-linked (Stratalinker 2400) and pre-hybridized for 3 h followed by 1-h hybridization with the quick hybridization solution (Stratagene, CA). The ^{32}P-dCTP labeled MADS-XI/GAPDH probe was made and purified as per manufacturer's protocol (Stratagene). The hybridization solution contained about 2×10^6 cpm/ml of the probe. The filter was then washed and exposed to X-ray film for 1–3 h and developed for autoradiography. Blotting experiments were repeated twice and the reproducible results were analyzed.

3. Results

The MADS-XI (224 bp) has a 68 bp unique sequence followed by a 156 bp sequence with 17 groups of varying number of repeats, interrupted by 1–10 nucleotides (Fig. 3). Blast and Blat search results showed high homology (98%) of this sequence with regions on chromosome 6q. However, when only dinucleotide repeats sequence (without the unique sequence) was blasted for homology search it showed high homology with the dinucleotide repeats on chromosomes 6, 16 and 17. Physical localization using radiation hybrid mapping however was not successful due to several non-specific PCR products with human and Chinese hamster ovary (CHO) template DNA samples.

Slot blot analysis of DNA from primary tumor cells and metastatic lymph nodes from five patients with ductal mammary carcinoma using the MADS-XI as a probe revealed its involvement in breast metastasis. As shown in the Fig. 4, the MADS-XI was present in the normal cells from all of the five patients while missing in four out of five (80%) metastasis cell DNA samples. In the patient number 3, however, the primary cell DNA band showed lesser (half) intensity suggesting the loss of heterozygosity.

VGATCTTAATCCGGGGAGTGGCGTATGTAGTAGAAGAGTCTGGATTTGAGTAG TGTATGGTAACGCCAG (T-G)$_5$ C (T-G)$_4$GTA (T-G)$_2$G (T-G)$_4$T(T-G)3 GCA(T-G)$_3$G(T-G)$_2$TATGG(T-G)$_3$G (T-G)$_3$A (T-G)$_4$GTGCG(T-G)$_2$AGATACGTGG(T-G)$_3$GGG(T-G)$_6$ G (T-G)$_3$ G (T-G)$_5$ GTA (T-G)$_2$ G (T-G)$_3$ GV.

Fig. 3. The complete MAGS-XI sequence enriched with TG repeats. Bold letters: unique sequence; V: vector sequence.

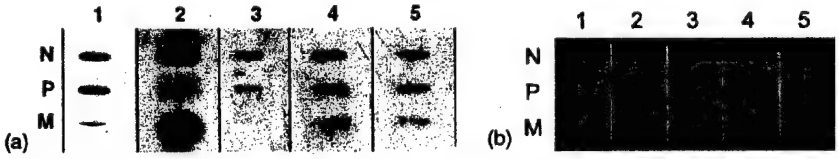

Fig. 4. Slot blot analysis of DNA samples from primary tumor and metastatic lymph node samples and matched normal cells from five patients (1, C-14153; 2, C-1050; 3, DS97-919; 4, C-14852; 5, DS-711). The first (N), second (P) and third (M) rows represent the DNA samples from normal, primary tumor and metastatic lymph node breast samples, respectively. (a) The blot is hybridized with ^{32}P-dCTP labeled MADS-XI probe; (b) the DNA loading is quantitated in the experimental blot by probing with GAPDH.

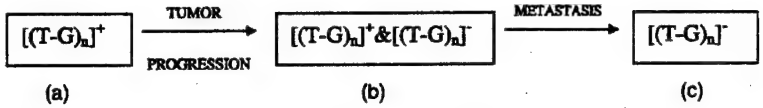

Fig. 5. Schematic representation of loss of T-G repeats with the progression of the disease. (a) Normal cells with $[(T\text{-}G)_n]^+$ repeats; (b) primary cells with $[(T\text{-}G)_n]^+$ and without $[(T\text{-}G)_n]^-$ repeats and (c) metastatic cells without any repeats $[(T\text{-}G)_n]^-$.

4. Discussion

Dinucleotide repeats are known to regulate the expression of several tumor/metastatic suppressor genes [11,13]. The loss of this MADS-XI in metastatic lymph node cell DNA samples from four out of five patients suggests its involvement in breast metastasis (Fig. 4). Complete loss of this sequence in lymph nodes, suggests that this sequence may be used as a diagnostic tool for breast lymph node metastasis. These findings are in support of our hypothesis (Fig. 5) that gradual loss of T-G repeats in tumor cells is associated with the progression of the disease which leads to the microsatellite instability (MSI) and/or loss of several tumor/metastasis suppressor genes in metastasis.

Blat search results indicated that both the whole MADS-XI sequence and only T-G repeat sequence from MADS-XI are matching with a sequence from chromosomes 6, 16 and 17 with high scores (98.7, 92, 93%). The unique sequence (68 bp) from MADS-XI, without T-G repeat sequence has matched only with chromosome 6q (98%). This may suggest that MSI due to loss of repeat sequences may cause allelic instability (AI) in several different chromosomes simultaneously. Our results implicate involvement of at least three chromosome regions in breast lymph node metastasis. They might also influence some important genes located in these three chromosomes (*IGF2R/M6PR*; 6q26, 6q27) [15,16], (*p53*; 17p13.1) [17,18], (*BRCA1*; 17q12–q21) [19]. Others also have reported the allelic loss of chromosomes 6, 16 and 17 because of MSI and AI during disease progression [20–23]. Furthermore, MSI causes replication error mismatch repair induction followed by mismatch repair gene mutation. Thus sum of these events probably activate the protooncogenes and inactivate tumor/metastasis suppressor genes leading to metastasis [24].

Currently we are screening circulating tumor cells with MADS-XI alone and in combination with other known makers to evaluate the state of the breast disease. The prediction for the genetic status of the circulating tumor cells will eventually convey the guidelines for early stage therapeutic modality to arrest or delay the progression of the disease [25].

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Title:
Expression Profile of Genes Associated with Antimetastatic Gene, nm23-Mediated Metastasis Inhibition in Breast Carcinoma Cells.

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Expression profile of genes in breast cancer metastasis

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The abbreviations used are:
nm23: non metastatic gene; EST: Expressed sequence tags; cDNA: complementary DNA.

Abstract

Metastases of various malignancies have been shown to be inversely related to the abundance of nm23 protein expression. However the downstream pathways involved in nm23 mediated suppression of metastasis have not been elucidated. In the present investigation, we used cDNA microarrays to identify novel genes and functional pathways in nm23 mediated spontaneous breast metastasis. Microarray experiments were performed in a pair of cell lines, namely: C-100 (only vector transfected; highly metastatic) and H1-177 (nm23 transfected; low metastatic), derived from human mammary carcinoma cell line, MDA-MB-435. The cDNA microarray analysis using GeneSpring software revealed significant as well as consistent alterations in the expression (up and down regulation) of 2,158 genes in a total of 18,889 genes between high and low metastatic cells. Some of these genes were grouped into 6 functional categories, namely invasion and metastasis, apoptosis and senescence, signal transduction molecules and transcription factors, cell cycle and repair, adhesion, and angiogenesis to extrapolate an association between these genes and different functional pathways involved in nm23 regulated metastasis. The results suggest that nm23 gene plays a major role in metastasis and its mechanism of action of metastasis suppression may involve down regulation of genes associated with cell adhesion, motility (integrins α -2, -8, -9, -L and -V, collagen type VIII α1, fibronectin 1, catenin, TGF β2, FGF7, MMP14 and 16, ErbB2), and possibly certain tumor/metastasis suppressors (2 members of SWI/SNF related matrix associated proteins 2 and 5 and PTEN).

Introduction

Reduced expression of nm23 gene is related to high incidence of lymph node and distant metastasis and to poor prognosis of patients in several human malignant tumors[1]. In some other cancers like prostate cancer, non-Hodgkin lymphomas and neuroblastoma, on the contrary, a high nm23-H1 expression is linked to an unfavorable outcome.[2] Recent studies have shown that in neuroblastoma, gain of 17q and activation of oncogene c-myc can upregulate nm23-H1 and -H2 and may confer a poor prognosis.[3] It is therefore possible that in these tumors, nm23 overexpression may be as a consequence of a different oncogenic or an unknown mechanism. While conflicting results were observed in different solid tumors, nm23, however, is universally classified as a putative metastasis suppressor gene. The nm23 expression pattern appears to be the key to the differences observed between tumor cells of high and low metastatic potential.[1] Experimentally it has also been demonstrated that nm23 may be involved in cellular functions leading to the metastatic phenotype, such as cell motility, which points to a regulatory role for nm23 proteins in cellular signaling pathways.[4,5]

While it has been shown that nm23 is involved in the suppression of metastasis, the exact mechanism by which it regulates metastasis remains to be elucidated. It is possible that nm23 activates or down-regulates certain target genes involved in the process of metastasis inhibition. To understand better the role of nm23 in the prevention of metastasis, we have compared gene expression profiles of a highly metastatic mammary carcinoma cell line, MDA-MB-435 with that of the same cell line transfected with nm23 that shows low metastasis. Gene expression comparisons were made by cDNA microarray analysis to determine the genes that are up-regulated and also those down-regulated by nm23 overexpression. We found that certain genes which are involved in adhesion and motility are responsive to overexpression of nm23. This is

the first study to elucidate the down-stream target gene(s) associated with nm23 at the global level, and also to suggest that prevention of metastasis is a commanding effort of nm23 by downregulating several target genes associated with motility and adhesion and possibly certain tumor suppressor genes.

Materials and Methods:

Cell lines and culture

A pair of breast cancer cell lines derived from MDA-MB-435 (a gift from Dr. P. Steeg, NCI/NIH) were used in this study. The nm23 gene transfected –435 cell line is named as H1-177 and empty vector transfected –435 cell line as C-100. While C-100 cell line is highly metastatic, the nm23 over expressing H1-177 cell line is known to be low metastatic. These cell lines were cultured in DMEM culture media (Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin in an atmosphere of 5% CO2 at 37°C. These were grown to 70–85% of confluence before being harvested for different experiments. Cells from the same passage (19^{th}) were counted and pooled for the RNA and protein expression experiments and also for *in vivo* Severe Combined Immunodeficiency (SCID) mouse studies to verify their metastatic potential.

Semiquantitative RT-PCR and western blotting analyses

These methods were followed as described in our previous publication.[6] In the semiquantitative RT-PCR, about 5µg of total cellular RNA was converted to cDNA, using Superscript II reverse transcriptase (Life Technologies, Inc.). The cDNA was diluted with water to a volume of 20µl. One µl of the diluted cDNA was used as template for each of the PCR reaction. The PCR conditions were optimized for each of the gene expression studied using Perkin-Elmer DNA thermal cycler. For each gene product, the PCR cycle numbers were adjusted such that the reaction fell within the linear range of product amplification. We designed the primer sets for the RT-PCR of some genes (Table 2). The β–actin was used as an internal control for loading. The PCR products were analyzed on 1% agarose gels and the products were photographed and quantitated by densitometry. For Western blotting analysis, protein was isolated from subconfluent cells[7] and electrophoresed (50µg/lane), transferred and blotted using primary antibody (anti-nm23, Ab-1, mouse monoclonal, 1:10 dilution, Oncogene Research Products, San Diego, CA) and secondary antibody (goat anti-mouse IgG HRP secondary antibody; 1:2000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA).

In Vivo metastasis assay

A mammary fat pad (MFP) SCID mouse model was used to evaluate the metastatic potential of these 2 human mammary carcinoma cell lines. MDA-MB-435 transfected with nm23 (H1-177) and vector only transfected cells (C-100) were injected (@$1X10^6$ cells) in a volume of 100 µl of DMEM without serum into the right sub-axillary MFPs of anesthetized 5- to 6-week-old female SCID mice. Five mice each were used for H1-177, C-100 and parental non-trasfected 435 cells as a positive control. Tumor sizes were monitored a week after inoculation of tumor cells. When the mean tumor diameter reached 1.0 cm, tumors were surgically removed. Six weeks later the animals were sacrificed and lungs were examined for visible metastatic foci and counted. Animals were maintained under the guidelines of the NIH and approved protocols of IACU Committee of AECOM.

cDNA microarray studies

cDNA microarray experiments were performed following our previously published protocols.[6,8]

Isolation of total cellular RNA

Total RNA was extracted using TRIzol Reagent (Life Technologies, Inc.) following our method reported previously.[8] Isolated RNA was electrophoresed through 1.0% agarose-formaldehyde gels to verify the quality. The concentration and purity of RNA were determined from absorbance measurements at 260 and 280 nm. For cDNA microarray experiments, RNA at a concentration of 7μg/μl with a purity of 1.8-1.9 (260/280 ratio) was used.

cDNA synthesis and microarray hybridization

One hundred μg of total cellular RNA (H1-177/C-100) was annealed to oligo(dT) and reverse-transcribed in the presence of Cy3-labeled (C-100) or of Cy5-labeled (H1-177) dUTP (Amersham Pharmacia Biotech, Piscataway, NJ), using 10,000 units/ml of Superscript II reverse transcriptase (Life Technologies, Inc.). The resulting Cy3- and Cy5-labeled cDNAs were treated with RNase One (Promega Corporation, Madison, WI) for 10 min at 37°C, combined, and purified by passing through a Centricon-50 filtration spin column (Millipore, Bedford, MA) to a final volume of 6.5 μl. The cDNA probes were then combined with 32.5 μl of hybridization solution and 1.0 μl of blocking solution to a final volume of 40 μl. The probe mixture was heated at 94°C for 2 min and centrifuged at 13,000 rpm for 10 min, and the supernatant was transferred to a clean tube and incubated at 50°C for 1 h before hybridizing to the microarrays. Hybridizations were performed on cDNA microarray glass slides prepared at the Albert Einstein College of Medicine microarray facility. Each slide (Chips: H7-119, 121 and 122) contained 20,000 unique human cDNA clones. The hybridization solution (40μl) was placed on a pretreated (UV cross linked) microarray slide, covered with a cover-glass (60mm), and then incubated in a hybridization chamber overnight at 50°C. After hybridization, the slide was washed at room temperature, first with 0.2XSSC, 0.1% SDS for 20 min with gentle shaking, and then with 0.2XSSC for two times (20 min each). The slide was dried by spinning at low speed (1000 rpm) in a centrifuge for 5 min, and stored in a dark box free from dust.

Scanning, gridding, and analysis

The slides were scanned using a Microarray Scanner 4000A (Axon Instruments Co., Foster City, CA) at the Albert Einstein College of Medicine Cancer Center microarray facility. The scanner output tif images were gridded and the fluorescence intensities of microarrays were then calculated using the GenePix 3.05 software. The final intensities represent the difference between average probe intensity and average local background intensity. Final intensities of green and red channels were filtered and the ratios of the red intensity to the green intensity were determined. cDNA microarray results were derived by comparing total RNA from 435 cells, highly expressing nm23 (Cy5-lebelled) *versus* 435 cells low expressing nm23 (Cy3-labelled). These experiments were repeated thrice and the results were analyzed using GeneSpring software (Silicon Genetics, Redwood City, CA). The average expression level of each gene in two groups was calculated and the cutoff value was set to 1.7 or 0.5 for the ratio of median. To generate the relative intensity (ratio) values each gene's measured intensity was divided by its control channel value in each sample. To correct for artifacts caused by non-linear rates of dye incorporation as well as inconsistencies of the relative fluorescence intensity between some red

and green dyes, we used an intensity-dependent normalization (known as Lowess Normalization). This normalization method fits a curve through the data and uses this curve to adjust the control value for each measurement. When the resulting normalized data is graphed versus the adjusted control value, the points are distributed more symmetrically around the 45° line. As mentioned elsewhere the AECOM made 20K cDNA chips were used in this investigation and the data was generated using Genepix program and analyzed by Genespring softwares. Red (H1-177) to green (C-100) ratios was analyzed in three completely independent experiments.

Results

Gene expression profile of highly metastatic breast carcinoma cell line was compared with that of nm23 mediated low metastatic cell line model by cDNA microarrays. The expression profile of different genes under different pathways/functions revealed the possible involvement of some known and many more unknown genes in nm23 directed breast metastasis inhibition.

Characterization of nm23 transfected cell lines

MDA-MB-435 breast carcinoma cell line is known to be highly metastatic (C-100) and the same transfected with nm23 gene, is low-metastatic (H1-177). However, these cell phenotypes are not distinguishable by morphological or growth rate variations. In order to avoid any confusion between these cell lines, before initiating the cDNA microarray experiments, we verified the expression of RNA and protein levels of nm23 gene in these cell lines, and also evaluated their metastatic potential in a SCID mouse model. RT-PCR (Fig. 3) and Western blotting (Fig. 1) results showed elevated levels of expression of mRNA (2.2 fold) and protein (20.5 fold) in H1-177 cell line compared to C-100. The results of experiments using the SCID mice (Fig. 2) to evaluate metastasis potential of these cell lines supported the working hypothesis that the nm23 overexpressing H1-177 cells were low metastatic compared to the C-100 cell line. As mentioned in the figure 2, the average number of metastatic cell foci were higher in C-100 (67 per mouse) compare to H1-177 (2 per mouse).

Genes associated with nm23 mediated breast metastasis

Expression of nm23 gene is known to reduce the metastatic potential in several carcinoma cell lines. However, downstream alterations in gene expression profile resulting from the modulation of nm23 gene especially at the global level, has not been studied. To determine the genes associated with nm23 mediated spontaneous breast metastasis inhibition, cDNA microarray experiments were performed using total RNA from two cell lines namely, C-100 (highly metastatic) and H1-177 (low metastatic). Out of 20,000 genes analyzed in each experiment due to printing and other technical problems, about 5.6% (1,111 out of 20,000) of these genes and ESTs were flagged and therefore were not considered for analysis in these experiments.

The cDNA microarray results, in 3 independent experiments, revealed 2158 genes, the expression (up and down regulation) of which was altered significantly and consistently between high and low metastatic cell RNA samples. The expression of 197 genes (1.04%) in the total of 18,889 cDNAs was elevated more than 1.7 fold in the low metastatic cells as compared to the highly metastatic cells. Of the 197 sequences, 133 (67.5% and 0.7% in total) are known genes while 64 (32.49% and 0.34% in total) are ESTs. Conversely, the expression of 1961 genes

(10.38%) was down regulated (< 0.5 fold) in the low metastatic cells or elevated (> 1.7 fold) in the highly metastatic cells. Of the 1961 sequences 811 (41.36% and 4.29% in total) are known genes while 1150 (58.64% and 6.10% in total) are ESTs. To extrapolate an association between these differentially expressed genes with different functional pathways of breast cancer metastasis, we grouped some of these genes and ESTs into 6 functional categories, namely invasion and metastasis, apoptosis and senescence, signal transduction molecules and transcription factors, cell cycle and repair, adhesion and angiogenesis. A partial list of genes are presented in table 1, under these 6 different groups. cDNA microarray expression levels of certain important genes associated with invasion and metastasis (nm23, PTEN, MMP1, SWI/SNF and ErbB2), adhesion (integrin α3 and fibronectin1), angiogenesis (VEGF) and cell cycle and DNA damage repair (ATM and BRCA2) in these 2 cell line RNA samples were validated by semi-quantitative RT-PCR (Fig. 3). Some of these gene expressions were also confirmed by real-time quantitative RT-PCR (data not shown).

Discussion

The discovery of nm23 gene has elicited a great deal of interest in understanding the molecular mechanisms involved in metastasis inhibition. Nm23 is considered a legitimate metastasis-suppressor gene in human breast cancer, since transfection of nm23-H1 into a breast cancer cell line suppressed metastasis by decreasing motility, invasion as well as colonization.[9] Human nm23 genes (H1 and H2) are shown to be identical to human nucleoside diphosphate kinases (NDPK)-A and B.[10] Although this observation attributed a biological function to nm23, no correlation between NDPK activity and the suppression of metastasis has been shown.[11,12] Thus very little is known about the exact role of nm23 in the process of metastasis inhibition.

To further elucidate the downstream pathways, identify novel genes and potential targets for drug development involved in nm23 mediated suppression of metastasis, we compared a pair of breast carcinoma cells with different metastatic potentials by cDNA microarray analysis. We found that nm23 transfection in low nm23 expressing cell line (MDA-MB-435) affected the expression of genes involved in a wide variety of biological functions as shown in table 1. Some of the identified genes and ESTs were not previously reported to be related with tumorigenesis and/or metastasis.

As shown in table 1, over expression of nm23 upregulated several genes associated with cancer, such as CDC 42, actin, integrin α3, C/EBP β and γ, cathepsin D, VEGF, MMP 1, rho G, angiogenin inhibitor etc. On the other hand, a large number of important genes associated with different aspects of tumor progression, such as cyclin F, CD2 associated protein, BRCA2, integrins (α 2, 8, 9, L and V), CDC2, CDK5, collagen type VIII α1, laminin, neuronal cell adhesion molecules, fibronectin 1, catenin, TGF β2, FGF7, MMP14 and 16, ErbB2 and also PTEN and 2 members of SWI/SNF related matrix associated proteins (2 and 5) are down regulated in low metastatic H1-177 cells. Thus these results provide a wide view of a large number of genes whose expression was altered due to over expression of metastasis suppressor gene, nm23 in the highly metastastic cell line (Table 1).

In our study, erb2 gene expression was down regulated in nm23 over expressing cells. Over expression of c-erb-2/her2/neu tyrosine kinase is linked to poor prognosis in breast cancer and an

increased risk of metastasis, and resistance to therapy.[13] While the mechanism of nm23 and erb2 interaction remains to be established, our study however clearly shows an inverse relationship between nm23 and erb2 expression. TGFβ and one of its receptor were down regulated following nm23 over expression indicating that nm23 is associated with TGFβ signaling pathway to prevent metastasis. In breast cancer, TGF-ß1 protein levels are augmented at the advancing edge of primary human breast carcinomas and in lymph node metastases.[14] Over expression of nm23 showed inhibition of cell motility in human and murine tumor cells.[15] In our study, nm23 over expression led to down-regulation of different integrins. In fact most of integrins were down-regulated by nm23 over expression, except for integrin alpha3, which was up-regulated by nm23 over expression. Besides integrins, other participants of cell adhesion and attachment, such as laminin and fibronectin were also down-regulated by nm23. The exact relationship of nm23 and α3 integrin remains to be seen, but many studies have linked α3-integrin to metastasis. In endometrium cancer, mammary carcinoma, and metastatic hepatocarcinoma, α3-integrin was found to be associated with tumor growth and metastasis.[16,17,18] VEGF expression level was found to be up-regulated in nm23 gene transfected cells. This is consistent with the finding that in thymic carcinoma, VEGF and nm23 expressions were strongly correlated.[19] Some proposed that angiogenesis and the expression of nm23-H1 protein together may play an important role in the lymphatic metastasis process of breast cancer.[20,21]

In summary, our studies demonstrate that nm23 gene plays a major role in metastasis and its mechanism of action of metastasis suppression in this cell line model, may involve down regulation of genes associated with cell adhesion, motility (integrins α -2, -8, -9, -L and -V, collagen type VIII α1, fibronectin 1, catenin, TGF β2, FGF7, MMP14 and 16, ErbB2), and tumor/metastasis suppressors (2 members of SWI/SNF related matrix associated proteins 2 and 5 and PTEN). Although the mechanism of action of nm23 in metastasis inhibition could not be established, this study however, highlights a list of genes that merit investigation in other metastatic cells and tumors.

Acknowledgements

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Table.1. Selected groups of genes that are differentially expressed in low metastatic cells (H1-177) compared to highly metastatic cells (C-100). Up regulation (>1.7 fold); down regulation (<0.5 fold).

Up regulated genes	Down regulated genes
Cell cycle and DNA damage repair	
1. W81196: CDC42 effector protein 2 2. AA634006: Actin, alpha 2	1. AA676797: Cyclin F 2. N48329: CD2-associated protein 3. AA488718: Human BRCA2 region, mRNA sequence CG018 4. AA041499: Cell division cycle 4-like 5. AA453176: Ataxia telangiectasia and Rad3 related 6. AA504348: Topoisomerase (DNA) II alpha (170kD) 7. AA598974: Cell division cycle 2, G1 to S and G2 to M 8. AA678295: BRCA1 associated RING domain 1 9. N73242: CHK1 (checkpoint, S.pombe) 10. AA954188: Tubulin-specific chaperone c 11. AA401479: Cyclin-dependent kinase 5
Apoptosis and senescence	
1. AA877255: Human interferon regulatory factor 7 2. AA058323: Interferon induced transmembrane protein 1 3. AA150500: Interferon stimulated gene (20kD)	1. N51014: Homo sapiens apoptotic protease activating factor 1 2. AA490894: Tumor necrosis factor receptor shedding aminopeptidase regulator type 1 3. AA293571: Tumor necrosis factor receptor superfamily, member 6 4. T50828: Caspase 7, apoptosis-related cysteine protease 5. AA699697: Tumor necrosis factor (TNF superfamily, member 2) 6. R37093: Apoptosis regulator
Signal transduction molecules and transcription factors	
1. H26183: CCAAT/enhancer binding protein (C/EBP), beta 2. AA676804: CCAAT/enhancer binding protein (C/EBP), gamma 3. AA419177: Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	1. AA102068: Heat shock transcription factor 4 2. AA234897: MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C) 3. AA039370: Transcriptional enhancer factor tef-1 4. N62394: Gap junction protein, beta 1, 32kD (connexin 32)
Angiogenesis	
1. R88242: Ribonuclease/angiogenin inhibitor 2. R19956: Vascular endothelial growth factor 3. AA664389: Transforming growth factor beta-stimulated protein TSC-22	1. AA598794: Connective tissue growth factor 2. N45138: Transforming growth factor, beta 2 3. H62473: Transforming growth factor, beta receptor III (betaglycan, 300kD)
Adhesion	
1. AA424695: Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor) 2. AA485373: Cathepsin D (lysosomal aspartyl protease)	1. R87964: Integrin, alpha 8 2. AA872420: Collagen, type VIII, alpha 1 3. AA463610: Integrin, alpha 2 4. R25521: Neuronal cell adhesion molecule 5. H24650: Laminin, gamma 1 6. AA865557: Integrin, alpha 9
Invasion and metastasis	
1. AA143201: Matrix metalloproteinase 1 (interstitial collagenase) 2. AA644092: Non-metastatic cell protein (NM23A) 3. R76314: Ras homolog gene family (rho G) 4. N72228: Synovial sarcoma, X breakpoint 2 5. AA411640: Ras-related GTP-binding protein 6. R50354: Leukemia inhibitory factor	1. W37864: Phosphatase and tensin homolog, PTEN 2. H24688: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2 3. AA598468: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a,member 5 4. N33214: Matrix metalloproteinase 14 5. AA443351: V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 6. H09997: Matrix metalloproteinase 16 7. AI002301: RAB13, member RAS oncogene family 8. N24966: V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3 9. W84711: Fibronectin1

Table 2. List of primers used for RT-PCR to validate the differential expression of some of the genes selected from cDNA microarray experiments.

Gene	Primers	Product (bp)
MMP-1	F: 5' TGTGGTGTCTCACAGCTTCC 3' R: 5' CACATCAGGCACTCCACATA 3'	250
α3-integrin	F: 5' TATTCCTCCGAACCAGCATC 3' R: 5' CTCTTCATCTCCGCCTTCTG 3'	250
NM23	F: 5' GCTGGTGAAGTACATGAACTC 3' R: 5' TCAGGCTTAAACCATAGGCTG 3'	221
VEGF	F: 5' CCTTGCTGCTCTACCTCCAC 3' R: 5' CACACAGGATGGCTTGAAGA 3'	197
ErbB2	F: 5' GAGCTAGAGCGGCTTTTGAA 3' R: 5' TGGTAGGACACCTCAAAGGG 3'	214
ATX	F: 5' GAGGTAAACCGCTATGGATTAC 3' R: 5' CCCTCAGAGGATTTGTCATCTC 3'	225
BRCA2	F: 5' GCTATCACAGGACCCAGACC 3' R: 5' TTACTTGAACCCGGGAAGTG 3'	197
SWI/SNF	F: 5' TCTTCTGCACTGGGGCTACT 3' R: 5' GGGAGACAGGGTTTTTGTCA 3'	199
Fibronectin	F: 5' ACCAACCTACGGATGACTCG 3' R: 5' GCTCATCATCTGGCCATTTT 3'	230
PTEN	F: 5' AGACCATAACCCACCACAGC 3' R: 5' ACACATAGCGCCTCTGACTG 3'	254
β-actin	F: 5' TCACCCACACTGTGCCCATCTACGA 3' R: 5' CAGCGGAACCGCTCATTGCCAATGG 3'	295

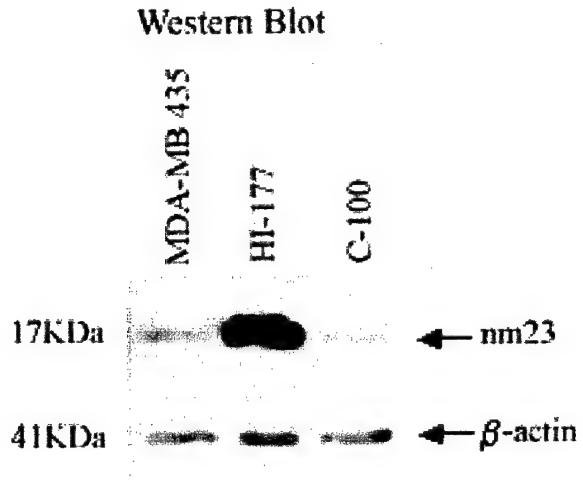

Fig. 1. Expression of nm23 in different MDA-MB-435 cell lines. Western blot showed about 20 fold overexpression of nm23 protein (17KDa) in H1-177 cells (low metastatic) compared to C-100 cells (highly metastatic). Same blot was stripped and probed with β-actin to demonstrate equal loading. As shown in figure 3, RT-PCR analysis showed 2.2 fold overexpression of nm23 transcript in H1-177 cell line compared to C-100.

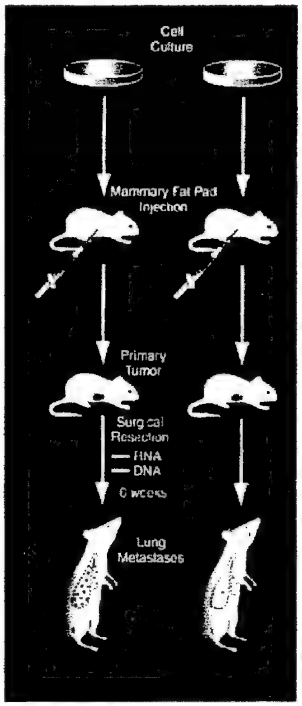

Fig. 2. *In Vivo* Spontaneous metastasis SCID mouse model: The left panel represents the mouse injected with C-100 cells (vector only) and right panel injected with the same cell line but transfected with nm23(H1-177). Mice injected with C-100 showed large number of metastatic foci (average number in 5 mice: 67) compared to that of H1-177 (average number in 5 mice: 2) supporting the hypothesis that nm23 transfected cells show reduction in lung metastasis.

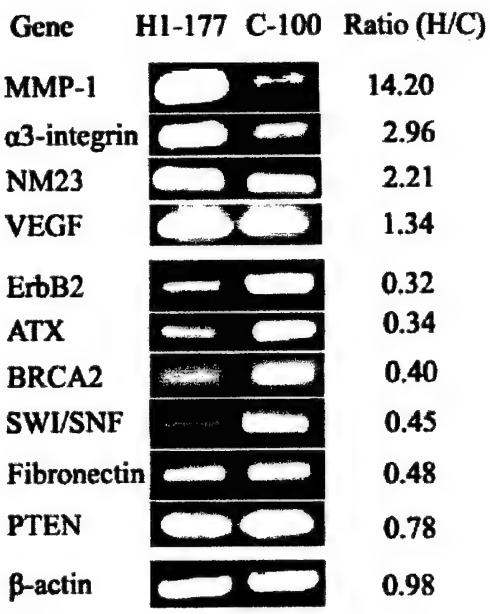

Fig. 3. Validation of cDNA microarray expression results of certain genes by RT-PCR.

[CANCER RESEARCH 61, 8022–8031, November 1, 2001]

Annexure IV

Altered Gene Expression Pattern in Cultured Human Breast Cancer Cells Treated with Hepatocyte Growth Factor/Scatter Factor in the Setting of DNA Damage[1]

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ABSTRACT

The cytokine hepatocyte growth factor/scatter factor (HGF/SF) protects epithelial and cancer cells against DNA-damaging agents via a pathway involving signaling from c-Met → phosphatidylinositol-3-kinase → c-Akt. However, the downstream alterations in gene expression resulting from this pathway have not been established. On the basis of cDNA microarray and semiquantitative RT-PCR assays, we found that MDA-MB-453 human breast cancer cells preincubated with HGF/SF and then exposed to Adriamycin (ADR), a DNA topoisomerase II inhibitor, exhibit an altered pattern of gene expression, as compared with cells treated with ADR only. [HGF/SF+ADR]-treated cells showed altered expression of genes involved in the DNA damage response, cell cycle regulation, signal transduction, metabolism, and development. Some of these alterations suggest mechanisms by which HGF/SF may exert its protective activity, *e.g.*, up-regulation of polycystic kidney disease-1 (a survival-promoting component of cadherin-catenin complexes), down-regulation of 51C (an inositol polyphosphate-5-phosphatase), and down-regulation of TOPBP1 (a topoisomerase IIB binding protein). We showed that enforced expression of the cdc42-interacting protein CIP4, a cytoskeleton-associated protein for which expression was decreased in [HGF/SF+ADR]-treated cells, inhibited HGF/SF-mediated protection against ADR. The cDNA microarray approach may open up new avenues for investigation of the DNA damage response and its regulation by HGF/SF.

INTRODUCTION

The cytokine HGF/SF[3] is a pleiotrophic mediator of multiple biological functions that plays significant roles in embryonic development, tissue and organ repair, tumorigenesis, and angiogenesis. HGF/SF has been found to protect various cell types against apoptosis induced by a variety of stimuli, including loss of contact with the substratum (1), exposure to staurosporine (a protein kinase inhibitor; Refs. 2, 3), and DNA damage (4–7). We have reported previously that various epithelial and carcinoma cell lines are protected by HGF/SF against apoptotic cell deaths induced by DNA-damaging agents, including ionizing radiation, ultraviolet (UV-C) radiation, and ADR (also known as doxorubicin; Ref. 5). ADR is a DNA intercalator and a DNA topoisomerase IIα inhibitor that induces single- and double-strand DNA breaks similar to those induced by ionizing radiation.

Interestingly, preincubation with HGF/SF also reduced the number of residual DNA strand breaks at 24 h after exposure to ADR or ionizing radiation, suggesting that HGF/SF may also enhance the rate of DNA repair (*i.e.*, strand rejoining; Ref. 6). The increased DNA repair and the cell protection against DNA damage appeared to be attributable to at least in part, to: (*a*) activation of a cell survival pathway involving PI3K and c-Akt (protein kinase B); and (*b*) subsequent stabilization of the protein levels of the antiapoptotic mitochondrial pore-forming protein Bcl-X_L (5, 6).

These studies have not revealed the downstream effector genes that mediate cytoprotection by HGF/SF. Cytoprotection by HGF/SF might involve nonnuclear events, such as inactivation of proapoptotic effectors (*e.g.*, Bad and caspase-9) by c-Akt-mediated protein phosphorylation events (8, 9). However, it might also involve prolonged patterns of altered gene expression induced by HGF/SF in the DNA-damaged cells. The latter possibility was suggested by the observation that maximal protection required a preincubation of cells with HGF/SF for at least 48 h before exposure to ADR (5). Shorter preincubation periods yielded less protection, and application of HGF/SF only at the time of ADR treatment and during the 72-h postincubation period gave no protection.

To investigate the potential alterations of gene expression that might contribute to HGF/SF-mediated cell protection, we have used a cDNA microassay approach, using a previously studied model for HGF/SF protection (5). MDA-MB-453 human breast cancer cells were preincubated with HGF/SF, exposed to ADR, and then postincubated in ADR-free culture medium for 72 h to allow the repair processes to proceed. Alterations of mRNA expression were examined in cells treated with [HGF/SF+ADR], in comparison with cells treated with ADR alone.

MATERIALS AND METHODS

Sources of Reagents and Vectors and Sources of Reagents and Antibodies. Recombinant human two-chain HGF/SF was generously provided by Dr. Ralph Schwall (Department of Endocrine Research, Genentech, Inc., South San Francisco, CA). ADR (doxorubicin hydrochloride) and MTT dye (thioazyl blue) were purchased from Sigma Chemical Co. (St. Louis, MO). Expression vectors encoding full-length and truncated or deleted forms of human CIP4 have been described earlier (10). These CIP4 cDNAs were cloned into the pRK5-myc mammalian expression vector, which provides an NH_2-terminal myc epitope tag.

Cell Lines and Culture. MDA-MB-453 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM supplemented with FCS (5% v/v), nonessential amino acids (100 mM), L-glutamine (5 mM), streptomycin (100 μg/ml), and penicillin (100 units/ml; all from BioWhittaker, Walkersville, MD). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

ADR Treatment. Subconfluent proliferating cells in 100-mm plastic dishes or 96-well plates were preincubated in the absence or presence of HGF/SF (100 ng/ml × 48 h) in serum-free DMEM and then sham-treated (control) or treated with ADR (10 μM × 2 h, at 37°C) in complete culture medium (DMEM plus 5% FCS). Cultures were then washed three times to remove the ADR and postincubated in fresh drug-free complete culture medium at 37°C for 72 h (again in the absence or presence of HGF/SF, respectively). Cultures were then harvested for isolation of total cell RNA and cDNA microarray or semiquantitative RT-PCR analyses.

Transient Transfections. Subconfluent proliferating cells were transfected overnight using Lipofectamine (Life Technologies, Inc., Rockville, MD; 10 μg of plasmid DNA/100-mm dish) and then washed to remove the excess vector

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[3] The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; ADR, Adriamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IP, immunoprecipitation; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-PCR.

and Lipofectamine. As a control for transfection efficiency, cultures were cotransfected with 10 μg of a β-galactosidase expression vector (pSV-β-gal; Promega Corp., Madison, WI) under parallel conditions; and β-galactosidase was detected using a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining kit (Gene Therapy Systems, Inc., San Diego, CA).

MTT Cell Viability Assay. This assay is based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt into an insoluble formazan precipitate, which is dissolved in dimethyl sulfoxide and quantitated by spectrophotometry (11). To test the effect of CIP4 on HGF/SF-mediated cell protection, cells transiently transfected with CIP4 expression vectors (see above) were harvested using trypsin and seeded into 96-well dishes (2000 cell/well) in standard growth medium, incubated for 24–48 h to allow attachment and entry into the cell cycle, preincubated ± HGF/SF (100 ng/ml × 48 h), treated with ADR (10 or 20 μM × 2 h), postincubated for 72 h, and tested for MTT dye conversion. Cell viability was calculated as the amount of MTT dye conversion relative to sham-treated control cells. Ten replicate wells were tested for each experimental condition. Statistical comparisons were made using the two-tailed Student's *t* test.

Isolation of RNA. After cell treatments ± ADR ± HGF/SF, the total cellular RNA was extracted using TRIzol Reagent (Life Technologies, Inc.), according to the manufacturer's instructions. The RNA was treated with DNase and precipitated using 95% ethanol prior to cDNA synthesis. Isolated RNA was electrophoresed through 1.0% agarose-formaldehyde gels to verify the quality of the RNA, and RNA concentrations were determined from absorbance measurements at 260 and 280 nm.

cDNA Synthesis and Microarray Hybridization. One hundred μg of total cellular RNA was annealed to oligo(dT) and reverse-transcribed in the presence of Cy3-labeled or of Cy5-labeled dUTP (Amersham Pharmacia Biotech, Piscataway, NJ), using 10,000 units/ml of Superscript II reverse transcriptase (Life Technologies, Inc.). The resulting Cy3- and Cy5-labeled cDNAs were treated with RNase One (Promega) for 10 min at 37°C, combined, purified by using a Centricon-50 filtration spin column (Millipore, Bedford, MA), and concentrated to a final volume of 6.5 μl. The cDNA was then combined with 12.5 μl of hybridization solution and 1.0 μl of blocking solution to a final volume of 20 μl. The mixture was heated at 94°C for 2 min and centrifuged at 13,000 rpm for 10 min, and the supernatant was transferred to a clean tube and incubated at 50°C for 1 h.

Hybridizations were performed on cDNA microarray glass slides prepared at the Albert Einstein College of Medicine microarray facility. Each slide contained 9216 unique human cDNA clones. The hybridization solution was placed on a pretreated microarray slide, covered with Hybri-slip, and then incubated in a hybridization chamber overnight at 50°C. After hybridization, the slide was washed at room temperature, first with 0.2 × SSC, 0.1% SDS for 20 min with gently shaking, and then with 0.2 × SSC two times (20 min each time). The slide was dried by spinning at low speed in a centrifuge for 5 min.

Scanning, Griding, and Analysis. The slides were scanned using a Microarray Scanner 4000A (Axon Instruments) at the Albert Einstein College of Medicine Cancer Center microarray facility. The scanner output images were localized by overlaying a grid on the fluorescent images, using the ScanAlyze software by Michael Eisen, Stanford University.[4] The fluorescent intensities were then calculated, using the program Copy of FUBAR! (the easy way out). The final reported intensity was the difference between average probe intensity and average local background intensity. Both final reported intensities (green and red) were filtered, and the spots with intensity <1.5 were eliminated. The ratios of the red intensity to the green intensity and green intensity to red intensity for all targets were determined. The cDNA microarray results comparing cells treated with (HGF/SF+ADR) *versus* ADR alone are based on three completely independent experiments involving separate cell treatments, separate RNA isolations, and separate microarray assays. The microarray results comparing cells treated with HGF/SF alone vs 0 (control) are based on two completely independent experiments.

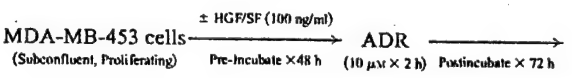
Semiquantitative RT-PCR Analysis. Aliquots of total cellular RNA (1.0 μg) were subjected to first-strand cDNA synthesis using Superscript II reverse transcriptase (Life Technologies, Inc.), and the cDNA was diluted five times with water. One μl of the diluted cDNA was used for each PCR reaction. PCR amplifications were performed using a Perkin-Elmer DNA thermal cycler. The PCR primer sets used in this study are shown in Table 1. The PCR reaction conditions were individually optimized for each gene product studied. For each gene product, the cycle number was adjusted so that the reactions fell within the linear range of product amplification. PCR reaction conditions and cycle numbers are shown in Table 2. The β-actin and β_2-microglobulin genes were used as controls for loading. PCR products were analyzed by electrophoresis through 1.2% agarose gels containing 0.1 mg/ml of ethidium bromide, and the gels were photographed under ultraviolet illumination. The amplified cDNA product bands were quantitated by densitometry.

IP and Western Blotting. Subconfluent proliferating cells were harvested, and whole cell extracts were prepared, as described earlier (5). Each IP was carried out using 6 μg of antibody and 1000 μg of total extract protein. Precipitated proteins were collected using protein G beads, washed, eluted in boiling Laemmli sample buffer, and subjected to Western blotting. The c-Met IP antibody was c-Met COOH-terminal antibody SP260 (Santa Cruz Biotechnology, Santa Cruz, CA). The control IP antibody was an equivalent quantity (6 μg) of normal mouse IgG (Santa Cruz Biotechnology).

Western blotting was performed as described earlier (5). The immunoprecipitated proteins or equal aliquots of total cell protein (50 μg/lane) were electrophoresed, transferred, and blotted using the appropriate primary antibody. The primary antibodies were: (*a*) anti-c-Met antibody H-190 (sc-8307, rabbit polyclonal IgG; Santa Cruz Biotechnology; 1:500 dilution); (*b*) anti-phosphotyrosine antibody (Ab-4, mouse monoclonal; Calbiochem/Oncogene Research Products; 1:500 dilution); and (*c*) an anti-myc mouse monoclonal antibody (Invitrogen, Carlsbad, CA) at a 1:1500 dilution, to detect the myc epitope tagged wild-type and mutant CIP4 proteins.

RESULTS

cDNA Microarray Analyses. The purpose of this study was to identify candidate genes, the expression of which is altered by HGF/SF in the setting of DNA damage, that might contribute to the HGF/SF-mediated protection against ADR. ADR is a DNA topoisomerase IIα inhibitor that induces single- and double-stranded DNA breakage. The basic experimental protocol is described in "Materials and Methods" and is summarized in the diagram shown below:



cDNA microarray analysis:
[HGF/SF + ADR] *versus* ADR

This design was chosen for several reasons. The main comparison was between [HGF/SF+ADR] *versus* ADR alone to identify genes for which expression was altered by HGF/SF during the response to DNA damage, because it is likely that some of these alterations may contribute to HGF/SF-mediated cell protection. However, a comparison of cells treated with HGF/SF *versus* CONTROL (sham treatment only) was also made. A postincubation period of $T = 72$ h after removal of ADR was used to examine well-established alterations in gene expression rather than transient changes occurring immediately after DNA damage. Furthermore, alterations in mRNA levels observed at $T = 72$ h are more likely to reflect changes in protein protein levels, because the mRNA alterations are of a prolonged duration.

Previous studies indicate that the ability of HGF/SF to protect cells against DNA-damaging agents is attributable to a c-Met receptor-mediated signaling pathway leading to the activation of a c-Akt-dependent survival pathway (6, 7). The HGF/SF-mediated cell protection was blocked by two fragments of the HGF/SF protein (designated NK1 and NK2) that bind strongly to the c-Met receptor, fail to fully activate c-Met signal transduction, and function as competitive antagonists of the full-length HGF/SF protein (5). Here, we show by IP-Western blotting that exposure of MDA-MB-453 cells to HGF/SF (100 ng/ml × 20 min) causes a large increase in the degree

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Table 1 *Primers used for semiquantitative RT-PCR analyses*

Gene name	Primer Sequences (5′ → 3′)	Genebank accession no.	Position in cDNA sequence	Expected size of product (bp)
ATM (ataxia telangiectasia mutated)	Sense: ctcagatggtcagaagtgttgaggc Antisense: tacactgcgcgtataagccaatcgc	NM_000051	8030–8757	728
Polycystic kidney disease-1 (PKD-1)	Sense: ctcctatcttgtgacagtcaccgcg Antisense: gtccagctgtaggagacgttggtgc	NM_000296	4528–5211	684
Lysyl hydroxylase (LH)	Sense: cgtcgatccctaattggccaggcc Antisense: aagatcgagctgtgcacagatgcc	L06419	2372–2986	615
Lactate dehydrogenase type A (LDH-A)	Sense: tagttctgccacctctgacgcacc Antisense: tataacacttggatagttggttgc	X02152.1	1330–1628	299
U1 snRNP70	Sense: cgcagatggcaagaagattgatggc Antisense: actccggctgcttcgccgcttccgg	NM_003089	1700–2096	397
Vascular endothelial growth factor (VEGF)	Sense: atgtctatcagcgcagctactgcc Antisense: caagctgcctcgccttgcaacgcg	XM_004512	150–548	399
Phosphoglycerate kinase 1 (PGK-1)	Sense: ggtagtccttatgngccacctaggc Antisense: cagccagcaggtatgccagaagcc	XM_010102	250–1011	762
c-Myc	Sense: cacatcagcacaactacgcagcgc Antisense: gactcagccaaggttgtgaggttgc	K02276	1331–1847	517
Cdc42-interacting protein (CIP-4)	Sense: caagacatggatgaacgcagg Antisense: gagatagtgcccicgctgg	AJ000414	688–1550	863
S100A9 (calgranulin B)	Sense: aggagttcatcatgctgatggcg Antisense: tggcctggcctcctgattagtgg	NM_002965	275–479	205
TNF-inducible gene product B94	Sense: gagtgcagtggcctggtcatggc Antisense: tcctgactcagcactgcagaggc	M92357	3306–3944	639
51C (inositol-5′-polyphosphate phosphatase like-1, INPPL1) Primer Set #1	Sense: cttccttcgattcagtgaggagg Antisense: ccttatcaatgctgatccactcg	L36818	2062–2804	743
51C (inositol-5′-polyphosphate phosphatase like-1, INPPL1) Primer Set #2	Sense: tcagggcagtatctctctgcc Antisense: accccaataatattaaggtgc	Y14385	4077–4522	446
Topoisomerase binding protein-1 (TOPBP1)	Sense: cgacctagagtacactaatcgc Antisense: gcttcctcattaaaccttgtgc	NM_007027	4630–5123	494
Protein Ser/Thr kinase STK2	Sense: caacttacagtgtgaaagctcgcc Antisense: cttaaggttattaacaatagcagg	NM_003157	2646–3144	499
Protein tyrosine phosphatase (PTPN2)	Sense: ctaaggaagacttatctcctgcc Antisense: tgtagcactgtcagttactagtg	NM_002828	938–1359	422
Human Gu protein	Sense: acaggcagagctggaaggac Antisense: actgatgcggtaggtacatc	U41387	1636–2123	488
β-Actin	Sense: tagcgggggttcacccacactgtgccccatcta Antisense: ctagaagcatttgcggtggacgatggaggg	XM_004814	541–1201	661
β_2-Microglobulin	Sense: ctcgcgctactctctctttc Antisense: tgtcggatggatgaaaccag	XM_007650	41–176	136

of activation (tyrosine phosphorylation) of c-Met (Fig. 1*A*). These findings support the role of the c-Met receptor in HGF/SF-mediated cell protection.

An illustration of cDNA microarrays comparing gene expression in cells treated with [HGF/SF+ADR] *versus* ADR alone and in cells treated with HGF/SF *versus* 0 (control) is provided in Fig. 1*B*. Gene products whose expression was consistently increased in [HGF/SF+ADR]-treated cells, relative to cells treated with ADR alone, by an average ratio of >1.7 in at least two of three completely independent experiments (*i.e.*, separate cell treatments, RNA isolations, and microarray hybridizations) are listed in Table 3. Those gene products for which expression was consistently decreased in cells treated with [HGF/SF+ADR] relative to ADR alone (ratio <0.7 in at least two of three completely independent experiments) are listed in Table 4. The ratio values shown in these tables represent the mean ± range ($n = 2$) or mean ± SD ($n = 3$). Some of the cDNA sequences contained on the microarray slides corresponded to expressed sequence tags for which the full-length sequence is not available in public domain databases. Alterations in the expression of cDNAs corresponding to these cDNAs, for which there is little or no information available on the structure-function of the putative gene product, are not included in Tables 3 and 4.

Although the HGF/SF-induced alterations in gene expression in the setting of DNA damage were not usually very large (1.7–4.0-fold increases and 0.41–0.67-fold decreases), these changes were reproducible. Elevated mRNA levels in the [HGF/SF+ADR] group (relative to ADR alone) were observed for various different functional classes of genes, including genes involved in the DNA damage response (*e.g.*, *ATM* and *FEN1*), cell cycle regulation (*e.g.*, *Hs-cul-3* and *HsGAK*), signal transduction (*e.g.*, *RHO B* and *CSBP1*), protein/RNA synthesis and metabolism (*e.g.*, *eIF3*, *U1*, and *snRNP70*), development and cellular differentiation (*e.g.*, *PKD1* and *IRX-2a*), general cellular metabolism (*e.g.*, LDH-A and PGK1), and other functional categories (see Table 3). The abbreviations for these gene products are defined, and their functions (or putative functions) are shown in Table 3.

Genes for which the mRNA levels were reproducibly decreased in [HGF/SF+ADR]-treated cells (relative to ADR alone) included those in similar functional classes: including DNA damage response (*e.g.*, *TOPBP1*), cell cycle regulation (*e.g.*, c-*Myc* and *CIP-4*), signal trans-

Table 2 *PCR reaction conditions for semiquantitative RT-PCR assays*

Gene name	PCR cycle parameters	No. of cycles
ATM	94°C (1 min); 65°C (1 min); 72°C (1 min)	31
PKD-1	94°C (1 min); 72°C (2 min)	28
Lysyl hydroxylase	94°C (1 min); 60°C (1 min); 72°C (1 min)	28
LDH-A	94°C (30 sec); 57°C (30 sec); 72°C (1 min)	25
U1 snRNP70	94°C (1 min); 60°C (1 min); 72°C (1 min)	30
VEGF	94°C (1 min); 57°C (1 min); 72°C (1 min)	28
Phosphoglycerate kinase-1 (PGK-1)	94°C (1 min); 60°C (1 min); 72°C (1 min)	25
c-Myc	94°C (1 min); 57°C (1 min); 72°C (1 min)	25
CIP-4	94°C (1 min); 59°C (1 min); 72°C (1 min)	35
S100A9	94°C (1 min); 65°C (1 min); 72°C (1 min)	31
B94	94°C (1 min); 65°C (1 min); 72°C (1 min)	31
51C [INPPL1] Primer Set #1 (743-bp)	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	33
51C [INPPL1] Primer Set #2 (446-bp)	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	30
TOPBP1	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	30
STK2	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	33
PTPN2	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	33
Human Gu protein	94°C (30 sec); 55°C (30 sec); 72°C (1 min)	30
β-Actin	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	23
β_2-Microglobulin	94°C (1 min); 54°C (1 min); 72°C (1 min)	28

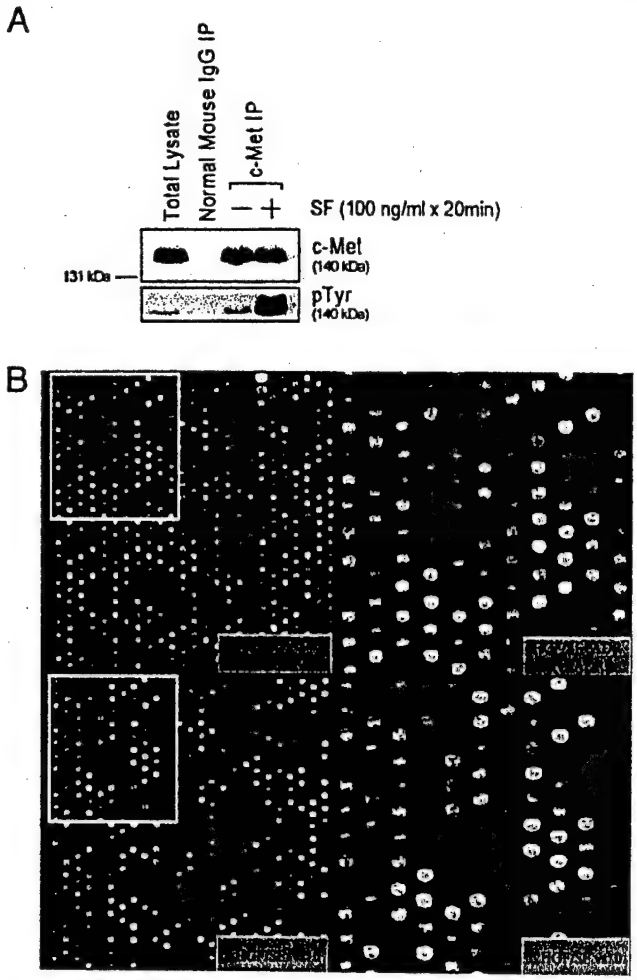

Fig. 1. HGF/SF activates c-Met and causes altered gene expression during DNA damage. *A*, HGF/SF causes activation (tyrosine phosphorylation) of the c-Met receptor. Subconfluent proliferating MDA-MB-453 cells were exposed to recombinant human HGF/SF (100 ng/ml × 20 min) and harvested for IP-Western blotting. Cells were immunoprecipitated using an anti-c-Met antibody or the same amount of normal IgG, as a negative control. IPs were then Western blotted using antibodies against c-Met and against phosphotyrosine residues. The basal level of phosphotyrosylated c-Met was low but was increased significantly by treatment with HGF/SF. No c-Met or phosphotyrosylated proteins were detected in the control (normal IgG) IP. *B*, illustration of cDNA microarray grids comparing gene expression in MDA-MB-453 cells treated with [(HGF/SF+ADR) *versus* ADR alone] (*top panels*) or with [HGF/SF *versus* 0 (control)] (*bottom panels*). Cells were treated as described in the text. The panels on the *right* show magnified views corresponding to the *boxed regions* of the array on the *left*. cDNAs isolated from cells treated with [HGF/SF+ADR] or HGF/SF were labeled with Cy5 (*red* dye), whereas cDNAs from cells treated with ADR alone or 0 were labeled with Cy3 (*green* dye). Spots showing *red* (or *green*) fluorescence correspond to genes overexpressed (underexpressed) in cells treated with [HGF/SF+ADR] relative to ADR alone and with HGF/SF relative to 0. *Yellow spots* correspond to genes equally expressed under the conditions being compared, whereas the absence of fluorescence indicates genes under either experimental condition. Note that alterations in gene expression, indicated by *red* or *green spots*, are more prominent in the comparison of [(HGF/SF+ADR) *versus* ADR] than [HGF/SF *versus* 0].

duction (*e.g.*, *51C* and *STK2*), and protein and RNA metabolism (*e.g.*, *human Gu protein*). Few or no gene products for which expression was reduced were observed in several functional classes, including development and differentiation, transcriptional regulation, and general cellular metabolism. However, in interpreting the significance of the lack of genes whose expression was decreased in certain functional classes, it should be noted that: (*a*) the number of genes included in each functional class is influenced by the ratio cutoffs, which is arbitrary; (*b*) fewer genes showed decreased than increased expression, based on the ratio criteria chosen; and (*c*) the inclusion of genes in the different functional categories was somewhat arbitrary, because some genes could be included in more than one category.

Table 5 shows a cDNA microarray comparison of gene expression in MDA-MB-453 cells treated with HGF/SF relative to untreated control cells. These data indicate that the number of genes whose expression is reproducibly altered and the magnitude of the alterations are relatively small when the experiment is performed in the absence of treatment with ADR. However, it was noted that *51C (INPPL1)*, which was decreased in [HGF/SF+ADR]-treated cells relative to ADR alone, was also decreased in HGF/SF-treated cells relative to control.

RT-PCR Assays. Because false-positive results are commonly observed in cDNA microarray analyses, we sought to confirm some of the gene expression alterations shown in Tables 3 and 4, via semiquantitative RT-PCR assays, using techniques described before by us (12, 13). The PCR primers and reaction conditions are provided in Tables 1 and 2, respectively. For each PCR assay, the reaction conditions and cycle numbers were individually optimized and adjusted so that the reaction fell within the linear range of product amplification. β-Actin and β_2-microglobulin, two genes whose expression was not altered, were used as controls for loading. The levels of amplified PCR products were quantitated by densitometry and expressed relative to β-actin. Figs. 2 and 3 show semiquantitative RT-PCR results for genes whose expression was either increased (Fig. 2) or decreased (Fig. 3) in cells treated with [HGF/SF+ADR] relative to ADR alone.

In general, qualitative agreement between the cDNA microarray and RT-PCR results was quite good, although there were differences in the quantitative extent of the gene expression alterations between the two assay methodologies. Figs. 2 and 3 show 16 different genes for which expression was either increased ($n = 7$) or decreased ($n = 9$) in [HGF/SF+ADR]-treated cells by both cDNA microarray and semiquantitative RT-PCR analyses. Genes confirmed to be increased in the [HGF/SF+ADR] group included: *ATM* (*ataxia-telangiectasia mutated*), *PKD1* (*polycystic kidney disease-1*), *lysyl hydroxylase*, *LDH-A* (*lactate dehydrogenase-A*), *U1 snRNP70* (*U1 small nuclear riboprotein*, M_r 70,000), *VEGF* (*vascular endothelial growth factor*), and *PGK1* (*phosphoglycerate kinase*). Genes confirmed to be decreased in the [HGF/SF+ADR] group included: c-*Myc*, *CIP4* (*cdc42-interacting protein-4*), *S100A9* (*calgranulin*), *B94* (a TNF-inducible gene product), *51C* (an inositol polyphosphate-5-phosphatase, also known as *INPPL1* and *SHIP-2*), *TOPBP1* (a DNA topoisomerase IIB binding protein), *STK2* (a serine/threonine protein kinase), *PTPN2* (a protein tyrosine phosphatase), and *Gu protein* (an RNA helicase).

Some of these alterations, although novel and not otherwise predictable, make sense within the context of explaining how HGF/SF may protect DNA-damaged cells, as will be considered in depth in the "Discussion." The down-regulation of *51C* in [HGF/SF+ADR]-treated cells was of particular interest because: (*a*) a decrease in *51C* mRNA levels was also noted in cells treated with HGF/SF alone (related to sham-treated control cells); and (*b*) *51C* is a lipid phosphatase, analogous to *PTEN*, except that *51C* removes the 5-phosphate whereas *PTEN* removes the 3-phosphate (14). Thus, *51C*, similar to *PTEN* (15), might be expected to inhibit c-*Akt* activation (see "Discussion"). Thus, we also examined *51C* expression levels by semiquantitative RT-PCR using a completely different set of primers. Similar results were obtained for *51C* using both sets of PCR primers (see Fig. 3).

Finally, it is noted that the RT-PCR assays provide additional information not obtained in the microarray comparisons. The RT-PCR assays allow comparisons of gene expression in cells treated with ADR, relative to control, a comparison not made by cDNA microarray

Table 3 *Genes whose expression is increased in [HGF/SF+ADR]-treated cells relative to ADR alone*

Gene name	Function	Ratio
DNA damage response		
ATM (ataxia-telangiectasia mutated)	DNA damage signaling, nuclear PI-3-kinase domain protein	2.9 ± 0.0
FEN1	Flap endonuclease-1, implicated in base excision repair pathway	2.1 ± 0.04
Cell cycle regulation		
CENP-F kinetochore	Microtubule motor protein, component of centromere	2.7 ± 0.3
Hs-cul-3	Homology to cullin/cdc53 family, ? role in cell proliferation control	2.4 ± 0.15
HsGAK	Unibquitously expressed perinuclear cyclin G-associated kinase	2.3 ± 0.4
NuMa gene (clone T33)	Nuclear mitotic protein, mitotic centromere function	2.1 ± 0.04
Cell growth regulator CGR19	Ring finger gene induced by p53	2.0 ± 0.2
Cyclin G2	May mediate proteolysis of G1 family cyclins	1.9 ± 0.2
Signal transduction-related		
RHO B transforming protein	Endosomal Rho protein, roles in receptor trafficking and apoptosis	4.0 ± 1.7
Dual specificity tyr phosphorylat. regulated kinase	Homolog of Drosophila kinase midbrain, ? role in brain development	2.1 ± 0.2
CSaids binding protein-1 [CSBP1]	Also known as p38, homolog of yeast Hog1 MAPK, stress response signaling	2.1 ± 0.15
Protein phosphatase PPP2R2A [PR53]	M_r 53,000 regulatory subunit of Ser/Thr protein phosphatase 2A	1.9 ± 0.2
RAB5A	ras-related small GTPase, regulator of vesicle trafficking	1.6 ± 0.1
Protein and RNA metabolism		
Lysyl hydroxylase [LH1, also known as PLOD]	Collagen modification, defective in Ehlers-Danlos syndrome VI	2.4 ± 0.7
eIF3	Eukaryotic translation initiation factor	2.2 ± 0.3
U1 snRNP70 (small nuclear ribonucleoprotein)	Associated with RNA processing and ubiquitination	2.1 ± 0.3
SAP49	Spliceosomal associated protein, RNA processing	2.1 ± 0.5
Cellular and nucleic acid binding protein		2.0 ± 0.3
SNC19	Putative novel human serine protease mapping to chr. 11q24-25	1.9 ± 0.2
β-COP	Golgi transport protein, component of COTI complex	1.8 ± 0.02
Cytokine and cytokine-induced		
Vascular endothelial growth factor (VEGF)	Stimulates endothelial cell proliferation and angiogenesis	2.8 ± 0.9
VEGF-related protein [VRP]	FLT4 ligand, VEGF family protein	2.1 ± 0.4
Interferon-induced M_r 17,000 protein	Precursor of 15 kDa protein homologous to ubiquitin	1.8 ± 0.2
Development and differentiation		
Keratin 17	Soft epithelial keratin 9 (*e.g.*, hair follicle)	3.8 ± 1.5
B4-2 protein	Proline-rich natural killer cell protein	2.6 ± 0.8
Keratin 19	Intermediate filament protein	2.5 ± 0.7
Iroquois class homeodomain protein IRX-2a	Transcription factor involved in embryonic patterning, regionalization	2.3 ± 0.4
Polycystic kidney disease-1 [PKD1]	Component of cadherin-catenin complex, endothelial survival	2.0 ± 0.4
Cancellous bone osteoblast	mRNA expressed in osteoblasts, function unknown	2.0 ± 0.1
SM22α homologue [TAGLN2]	Marker of differentiated smooth muscle (SM)-like cells	1.7 ± 0.2
Transcriptional regulation		
RIP140	Nuclear receptor-interacting protein, transcriptional coactivator	2.1 ± 0.1
hkf-1	Novel zinc finger protein isolated from a brain cDNA library	2.1 ± 0.0
DGS-I	DiGeorge (velocardiofacial) syndrome candidate gene	2.0 ± 0.45
General cellular metabolism		
Lactate dehydrogenase-A [LDH-A]	Enzyme involved in anaerobic glycolysis	4.1 ± 1.0
Phosphoglycerate kinase [PGK1]	Glycolytic enzyme, induced by hypoxia-inducible factor HIF-1	4.0 ± 1.4
Hexokinase-1	Early glucose metabolic enzyme	2.2 ± 0.3
Glucosylceramidase precursor	Degradation of GlcCer, mutated in Gaucher's disease	2.0 ± 0.4
Phosphoglycerate mutase 1 [PGAM1]	Late glycolytic pathway enzyme	1.7 ± 0.25
Cytoskeletal and structural proteins		
Ezrin-radixin-moesin phosphoprotein 50 [EBP50]	PDZ phosphoprotein, linkage of cell membrane to cytoskeleton	3.8 ± 1.0
p16-Arc [ARC16]	Arp 2/3 complex subunit, control of actin polymerization	1.8 ± 0.1
Miscellaneous and unknown function		
XAP-5	Unknown function	3.1 ± 0.7
OriP binding protein [OBP1]	Binds to Epstein Barr virus replication origin	2.1 ± 0.2
JTV-1	Gene overlapping PMS2, function unknown	1.9 ± 0.2
MAC30 (3' end)	Meningioma expressed protein	1.9 ± 0.2
Sm-like (CaSm)	Cancer-associated Sm motif-like domain protein	1.7 ± 0.2

analysis. Thus, in Fig. 2, it was observed that in most cases, the main effect of HGF/SF was not to alter gene expression by itself but to block the ADR-induced reduction of mRNA levels that were observed in the absence of HGF/SF. In Fig. 3, with the exception of *51C* and *PTPN2*, HGF/SF by itself did not significantly alter gene expression; but its main effect was to block the ADR-induced up-regulation of mRNA levels. However in some cases, the mRNA levels in [HGF/SF+ADR]-treated cells were reduced to below control levels (*e.g.*, *CIP4* and *TOPBP1*).

Role of CIP4 in HGF/SF-mediated Protection against ADR. The cdc42-interacting protein-4 (CIP4) was originally identified as a protein that binds to the activated form of cdc42, a Rho-like small GTPase, and was subsequently found to bind to the Wiskott-Aldrich syndrome protein (WASP) through its COOH terminus and to microtubules through its NH_2 terminus (Refs. 10, 16; illustrated in Fig. 4*A*). Although CIP4 is not known to be involved in cell survival or apoptosis pathways, the finding that CIP4 mRNA expression is up-regulated by ADR and that HGF/SF blocks the ADR-induced up-regulation of CIP4 raises this possibility. To determine whether CIP4 could modulate the survival of MDA-MB-453 cells in response to of ADR or HGF/SF, MDA-MB-453 cells were transfected with expression vectors encoding wild-type (wt) or mutant (truncated or deleted) forms of CIP4 containing an NH_2-terminal myc epitope tage and then assayed for their survival response. The MTT assay, which measures cytotoxicity as the loss of mitochondrial function (*i.e.*, the ability to reduce a tetrazolium dye to formazan) was used to quantitate cell viability (11). Expression of these proteins was confirmed by Western blotting of transfected cells using an anti-myc antibody (see Fig. 4*B*).

Cells transfected with wild-type CIP4 (wtCIP4) showed an increased sensitivity to ADR, as well as a significantly decreased degree of cytoprotection by HGF/SF (Fig. 4*C*), consistent with a role as a modulator of DNA damage or apoptosis response pathways. In the absence of HGF/SF, the decrease in cell survival (viability) in wt-CIP4-transfected cells (relative to the empty vector transfected control) treated with ADR alone was greater at 10 μM ADR (−28%; $P < 0.001$, two-tailed t test) than at 20 μM ADR (−10%; $P < 0.05$,

Table 4 *Genes whose expression is decreased in [HGF/SF+ADR]-treated cells, relative to ADR alone*

Gene name	Function	Ratio
DNA damage response		
P glycoprotein 3/MDR3 [PGY3]	Homologue of multidrug resistance protein MDR-1, drug transport	0.51 ± 0.07
Topoisomerase binding protein-1 [TOPBP1]	BRCT domain protein, binds DNA topoisomerase IIB	0.61 ± 0.05
Cell cycle regulation		
c-Myc	Proto-oncogene, functions in growth, differentiation, apoptosis	0.41 ± 0.04
CIP4 (cdc42-interacting protein)	Interacts with Wiskott-Aldrich protein, localized in cytoskeleton	0.42 ± 0.08
ras inhibitor (3' end)	Effector or regulator of H-Ras activity	0.60 ± 0.07
Signal transduction-related		
M_r 180,000 transmembrane PLA2 receptor	Receptor for secretory phospholipases A2, internalizes PLA2	0.51 ± 0.05
Protein tyrosine phosphatase PTPN2	Also known as PT PTP (T cell protein tyrosine phosphatase)	0.57 ± 0.17
Proto-oncogene c-*mer* [MERTK]	Member of Axl subfamily of receptor tyrosine kinases	0.57 ± 0.10
Protein serine/threonine kinase STK2	Homologue of cell cycle regulatory kinase NIMA	0.60 ± 0.05
51C [INPPL1]	Inositol polyphosphate-5'-phosphatase-like (also known as SHIP-2)	0.61 ± 0.10
Apoptosis-related		
CD40L receptor	Receptor for CD154, member of TNF death receptor family	0.57 ± 0.08
Protein and RNA metabolism		
Human Gu protein	RNA helicase, member of DEXD box family, target of adriamycin	0.46 ± 0.01
Cathepsin K precursor	Lysosomal acid cysteine protease, mediates proteolysis of bone	0.49 ± 0.10
Cytokine and cytokine-induced		
B94	TNF-induced gene product, unknown function	0.56 ± 0.07
Tazarotene-induced gene 2 [TIG2]	Novel retinoid-responsive gene, deficient in psoriatic skin	0.57 ± 0.02
IGF-1 (somatomedin-C)	Insulin-like growth factor-1	0.58 ± 0.02
FGF-7 (fibroblast growth factor-7)	Also known as keratinocyte growth factor, epithelial-specific growth factor	0.59 ± 0.07
Development and differentiation		
None		
Transcriptional regulation		
None		
General cellular metabolism		
None		
Cytoskeletal and structural proteins		
S100A9 (calgranulin B)	Secretory protein, ? roles in inflammation, eicosanoid metabolism	0.42 ± 0.25
Human triadin	Integral membrane protein, binds calsequestrin	0.58 ± 0.16
Vascular cell adhesion molecule VCAM1	Ig superfamily, interacts with α-4 integrins, cell trafficking	0.58 ± 0.07
Ankyrin G	Axon nodal protein involved in assembly of specialized structures	0.59 ± 0.13
Miscellaneous and unknown function		
hORC2L (origin recognition complex)	Putative replication initiation protein	0.58 ± 0.05
CHD2	Chromodomain helicase DNA-binding protein 2	0.60 ± 0.13
Rip-1 (Rev-interacting protein)	Interacts with HIV Rev protein, ? function	0.67 ± 0.01

two-tailed *t* test). This finding might reflect a greater degree of up-regulation of endogenous CIP4 expression at the higher dose of ADR, so that the transfected wtCIP4 has a smaller effect. For cells treated with HGF/SF, at both 10 and 20 μM ADR, the survival of the wtCIP4-transfected cells was significantly lower than the empty vector-transfected cells ($P < 0.001$).

The quantitative degrees of cell protection by HGF/SF were calculated based on the following equation, where (S/S_o) = cell viability relative to control:

$$\text{Protection by HGF/SF (\%)} = \{[(S/S_o)_{+\text{HGF/SF+ADR}} - (S/S_o)_{0\ \text{HGF/SF+ADR}}]/[(S/S_o)_{0\ \text{HGF/SF, 0ADR}} - (S/S_o)_{0\ \text{HGF/SF+ADR}}]\} \times 100$$

The % protection values at doses of 10 and 20 μM ADR were averaged and plotted in the bottom panel of Fig. 4*C*. On the basis of these calculations, transfection of wtCIP4 reduced the HGF/SF-mediated cell protection from ~85 to 40%. On the other hand, there was no effect of wtCIP4 on cell viability in the absence of ADR (100% of control).

Expression vectors encoding mutant forms of CIP4 included a deletion of the microtubule binding domain (CIP4 118–545), a deletion missing the cdc42 binding region (CIP4 Δ 383–481) and a deletion of the COOH-terminal WASP binding domain (Fig. 4*A*). In general, these deletion mutants had little or no effect on the degree of HGF/SF-mediated cell protection, nor did they affect cell viability in the absence of ADR (Fig. 4*C*). However, cells transfected with the mutant CIP4 cDNAs did show an increase in cell viability (by ≅15–20%) at 20 μM ADR in the absence of HGF/SF. This finding may be attributable to their function as dominant inhibitors of the endogenous wild-type CIP4, although that conclusion cannot be made from this experiment alone.

Similar findings were obtained using another cell type that is also

Table 5 *Genes whose expression is altered in HGF/SF-treated cells, relative to untreated control cells*

Gene name	Function	Ratio
Gene products increased in HGF/SF-treated cells		
Interleukin-8 (IL-8)	Proinflammatory & angiogenic cytokine, neutrophil chemotaxis	1.6 ± 0.01
(Clone ch13 lambda 7) α-tubulin	Microtubule protein	1.6 ± 0.02
Cytochrome c oxidase VIIc subunit [COX7C]	Subunit of COX holoenzyme, mitochondrial energy production	1.5 ± 0.01
Tubulin β-1 chain	Microtubule protein	1.5 ± 0.01
Gene products decreased in HGF/SF-treated cells		
51C [INPPL1]	Inositol polyphosphate-5'-phosphatase-like (aka. SHIP-2)	0.43 ± 0.0
il-TMP (intestine/liver tetraspan protein)	Integral membrane protein, density-dependent growth regulation	0.57 ± 0.13
Integrin α-8 subunit, 3' end	Integrin expressed in developing brain and mesangial cells	0.58 ± 0.02
Topoisomerase IIB [TOP2B]	Nuclear enzyme involved in DNA replication and transcription	0.65 ± 0.01
Corticotrophin releasing factor receptor precursor	Mediates release of corticotrophin (ACTH)	0.69 ± 0.01
Osteoblast mRNA for ostenidogen	Basement membrane component, entactin/nidogen family	0.69 ± 0.05
Janus kinase 1 [JAK1]	Mediates tyrosine phosphorylation of STAT1	0.70 ± 0.01
MutS homologue 3 [MSH3]	DNA mismatch repair enzyme	0.72 ± 0.01

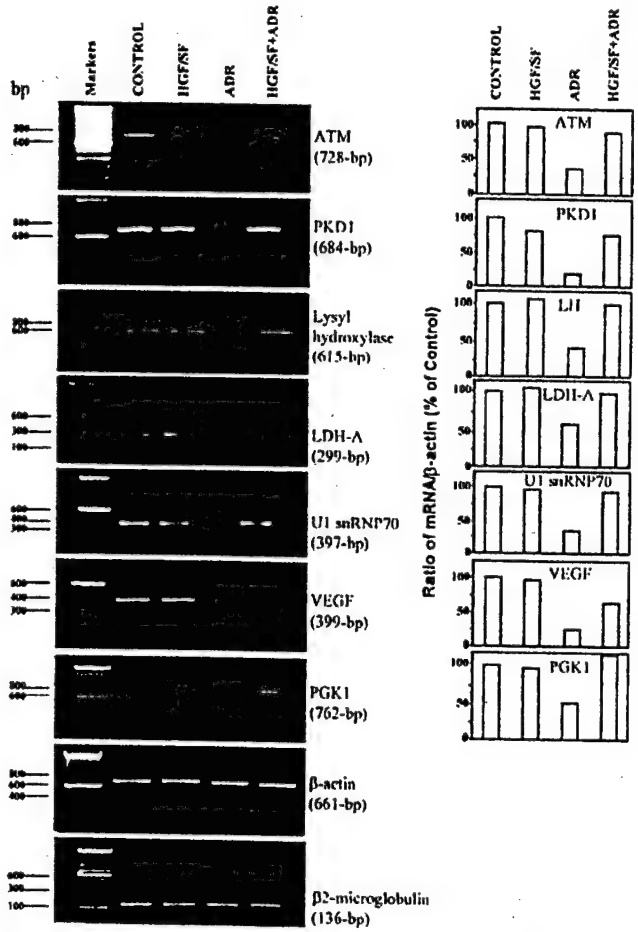

Fig. 2. Semiquantitative RT-PCR analyses of genes for which expression was increased in MDA-MB-453 cells treated with [HGF/SF+ADR] relative to ADR alone. Subconfluent proliferating cells were preincubated ± HGF/SF (100 ng/ml × 48 h), treated ± ADR (10 μM × 2 h), washed three times to remove the ADR, and postincubated for 72 h in fresh drug-free medium, as described in the text. RNA was collected, and RT-PCR assays were performed (see "Materials and Methods" and Tables 1 and 2 for methodological details). β-Actin and β_2-microglobulin were used as controls for loading. The amplified PCR products were quantitated by densitometry and expressed relative to β-actin, as a percentage of the control (0 HGF/SF, 0 ADR).

protected against ADR-induced DNA damage by preincubation with HGF/SF, DU-145 human prostate cancer cells (6). Thus, wtCIP4, but not the mutant or truncated forms of CIP4, blocked the HGF/SF-mediated protection against ADR (data not shown). These findings are consistent with a role for CIP4 as a regulator or modulator of cell survival in the setting of DNA damage.

DISCUSSION

These studies revealed an interesting pattern of up-regulation and down-regulation of genes in MDA-MB-453 cells treated with [HGF/SF+ADR], as compared with ADR alone. Admittedly, some of these gene products may be altered simply because of the higher proportion of surviving cells in the [HGF/SF+ADR]-treated group relative to the ADR-treated group. Gene products of this type might include lactate dehydrogenase [LDH-A] and phosphoglycerate kinase [PGK1], which were increased in [HGF/SF+ADR]-treated cells. However, the complexity of the findings, including many genes that were either increased or decreased in ADR-treated cells, suggest a more selective pattern of altered gene regulation.

We have reported previously that in addition to protecting cells against cytotoxicity and apoptosis induced by DNA damage, HGF/SF enhanced the ability of carcinoma cells, including MDA-MB-453 cells, to repair DNA strand breaks induced by ADR or X-rays (6). The observation that cells treated with [HGF/SF+ADR] show altered expression of certain gene products involved in DNA damage response pathways is consistent with that prior finding. For example, *ATM* (*ataxia-telangectasia mutated*), a nuclear protein kinase involved in DNA damage signaling (17), and *FEN1* (*flap endonuclease-1*), an enzyme implicated in the base excision repair pathway (18), were up-regulated in [HGF/SF+ADR]-treated cells. A mutation or deletion of the *ATM* gene leads to a defect in the repair of double-strand DNA breaks and increased sensitivity to ionizing radiation.

We also found that ADR caused the down-regulation of the *PKD1* (*polycystic kidney disease-1*) gene product, and HGF/SF blocked the ADR-induced down-regulation of *PKD1* expression. *PKD1* has been identified as a developmentally regulated gene, the absence of which is linked to type I autosomal dominant polycystic kidney disease (19). The function of this gene is not well understood, but *PKD1* was found to encode a large cell membrane protein associated with the cadherin-catenin cell:cell adhesion complex (20). Interestingly, the PKD1 gene product was shown recently to play roles in maintaining the structural integrity of blood vessels (21) and in protecting MDCK epithelial cells against apoptosis (22). We had reported previously that HGF/SF

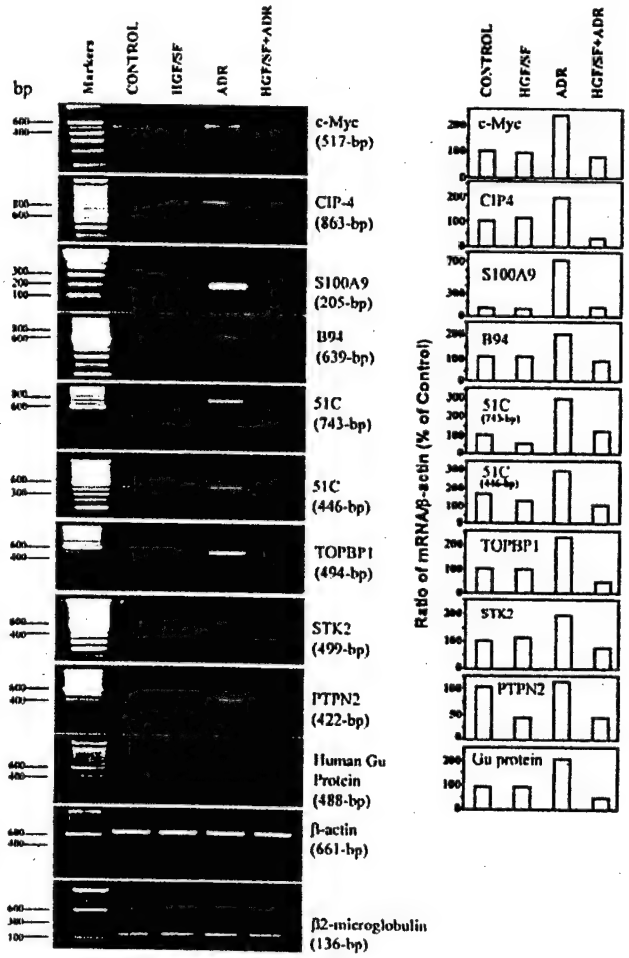

Fig. 3. Semiquantitative RT-PCR analyses of genes for which expression was decreased in MDA-MB-453 cells treated with [HGF/SF+ADR] relative to ADR alone. Assays were performed as described in the Fig. 2 legend. Note that 51C was analyzed using two completely different sets of PCR primers.

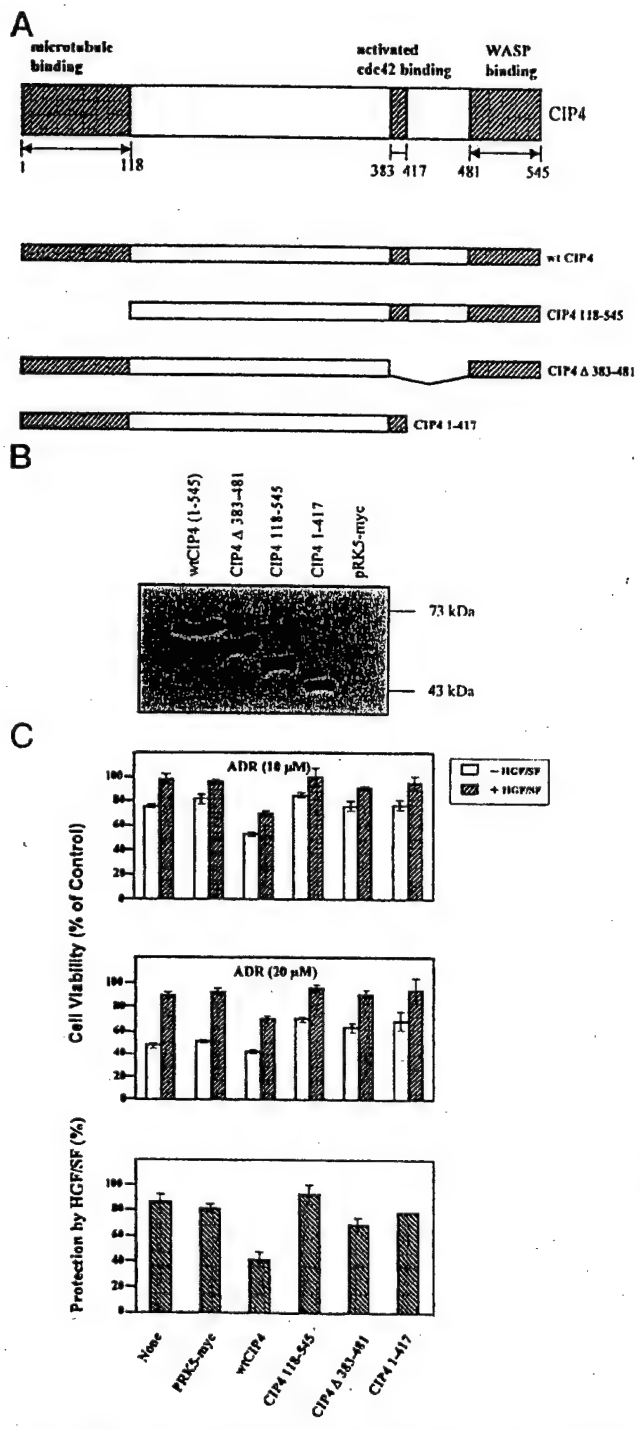

Fig. 4. Effect of genetic manipulation of cdc42-interacting protein (CIP4) expression on HGF/SF-mediated protection of MDA-MB-453 cells. *A*, schematic diagrams of CIP4 expression vectors. The human CIP4 cDNAs were cloned into the pRK5-myc mammalian expression vector, which provides an NH_2-terminal myc epitope tag. *B*, expression of wild-type and mutant CIP4 proteins. Cells were transfected with the different CIP4 expression vectors as described below (*C*), and the dishes were incubated for 24 h to allow expression of the encoded proteins. Proteins of the expected sizes were detected by Western blotting, using an antibody against the myc epitope tag. Cells transfected with the empty pRK5-myc vector showed no myc-tagged proteins. *C*, effect of transient expression of wild-type (wt) and mutant CIP4 proteins on HGF/SF-mediated cell protection. Subconfluent proliferating cells in 100-mm dishes were transiently transfected overnight with 10 μg of each vector, in the presence of Lipofectamine. Cells were washed, subcultured into 96-well dishes, pre-incubated ± HGF/SF (100 ng/ml × 48 h), exposed to ADR (10 or 20 μM × 2 h), washed, postincubated for 72 h in fresh drug-free medium, and assayed for MTT dye conversion. Cell viability values (means; *bars*, SE) are based on 10 replicate wells. For each experimental condition, cells treated with [HGF/SF + ADR] showed higher viability than those treated with ADR alone ($P < 0.001$, two-tailed t test). The viability of cells transfected with wtCIP4 and treated with [HGF/SF+ADR] was significantly reduced, compared with similarly treated untransfected or empty vector-transfected cells ($P < 0.001$).

protects both vascular endothelial and MDCK epithelial cells against DNA damage-induced apoptosis (4, 5). Thus, inhibition of the down-regulation of *PKD1* by HGF/SF may be a cytoprotective function, one which merits further investigation.

On the other hand, the expression of the topoisomerase binding protein TOPBP1, which binds DNA topoisomerase IIB and also shows DNA strand break binding activity (23–25), was decreased in cells treated with [HGF/SF+ADR]. ADR causes DNA strand breakage in part by converting the DNA topology enzyme topoisomerase II into a DNA cleaving enzyme (26). It is thought that topoisomerase binding proteins such as TOPBP1 may contribute to or potentiate ADR-mediated DNA damage, but the role of TOPBP1 in this process remains to be established. The finding that ADR up-regulates TOPBP1 expression and that the up-regulation is blocked by HGF/SF is provocative, because it suggests a potential mechanism by which HGF/SF might modulate the DNA damage and repair process, upstream of DNA-damage induced apoptosis. HGF/SF blocked the ADR-induced up-regulation of the human Gu protein. Gu is a DEXD box nucleolar RNA helicase, which presumably participates in aspects of RNA synthesis and processing (27). This finding is interesting because recent evidence suggests that, like topoisomerase II, Gu may be a target of ADR (28). However, the significance of this finding relative to HGF/SF-mediated cell protection remains to be determined.

A number of gene products implicated in signal transduction pathways were found to be up-regulated (*e.g.*, RhoB and RAB5A) or down-regulated [*e.g.*, STK2 (a serine/threonine kinase), PTPN2 (also known as T-cell protein tyrosine phosphatase, TCPTP) and 51C (also known as INPPL1 or SHIP-2)]. Expression of the *51C* gene, which encodes an the inositol polyphosphate-5-phosphatase (29), was decreased in both HGF/SF-treated cells (relative to control) and [HGF/SF+ADR]-treated cells (relative to ADR alone). This finding is of particular interest because of previous studies demonstrating a requirement for PI3K → c-Akt signaling in the HGF/SF-mediated protection of breast cancer (MDA-MB-453) and glioma cell lines against apoptosis (6, 7, 30).

It had been reported previously that the tumor suppressor PTEN/MMAC1, an inositol polyphosphate-3-phosphatase, inhibited the PI3K/Akt pathway through its lipid phosphatase activity (15). Recently, *51C* was similarly found to act as an inhibitor of the PI3K/Akt pathway, presumably also by reducing the levels of phosphatidylinsitol-3,4,5-phosphate [$PI(3,4,5)P_3$], which is generated through the lipid kinase activity of PI3K (31). Thus, the reduced expression of 51C in HGF/SF-treated cells should have the effect of maintaining the levels of $PI(3,4,5)P_3$, which is essential for the activation and proper localization of c-Akt.

Interestingly, it has been demonstrated that one of the splice variants of the protein tyrosine phosphatase PTPN2/TCPTP, TC45, can inhibit epidermal growth factor receptor-mediated activation of PI3K/c-Akt signaling (32). Although the role of PTPN2 in c-Met receptor signaling and the important *in vivo* substrates for PTPN2 are unclear, the finding that HGF/SF down-regulates *PTPN2* gene expression again raises the possibility that PTPN2 is a target for the HGF/SF-mediated protection against DNA-damaging agents.

A cytoskeleton-associated cdc42-interacting protein, CIP4, was found to be up-regulated in ADR-treated cells, whereas HGF/SF blocked the up-regulation of CIP4. The function of CIP4 has not been

established definitively, but CIP4 may function, in part, to carry the Wiskott-Aldrich syndrome protein (WASP), a multidomain protein involved in cytoskeletal organization, from actin filaments to microtubules (10). We showed that forced expression of wild-type human CIP4 reduced the degree of HGF/SF-mediated protection of MDA-MB-453 cells to 50% or less of that observed in untransfected or empty vector-transfected control cells. On the other hand, expression of internally deleted or truncated CIP4 proteins did not inhibit cell protection. These findings suggests a role for CIP4 in cell survival/apoptosis pathways, a finding that is not obvious based on its known activities and protein interactions.

Although we have focused on some of the more novel findings of this study, not all of the cDNA microarray and RT-PCR results were unexpected. For example, the finding that ADR up-regulates c-Myc mRNA expression and that the up-regulation was blocked by HGF/SF was not unexpected. We reported similar results based on Western blotting of MDA-MB-453 cells (5). The transcription factor c-Myc has been implicated in a variety of cellular processes, including proliferation, differentiation, transformation, and apoptosis. Overexpression of c-Myc renders cells more susceptible to apoptosis through both p53-dependent and p53-independent mechanisms (33, 34). Thus, theoretically, down-regulation of c-Myc by HGF/SF in the setting of DNA damage might be expected to confer protection against apoptosis.

We had also reported that ADR down regulates the protein levels of the antiapoptotic protein Bcl-X_L, whereas HGF/SF blocks the ADR-induced downregulation of Bcl-X_L protein in MDA-MB-453 cells (5). Bcl-X_L was not present among the cDNAs spotted onto the microarrays slides used in this study. However, we examined the Bcl-X_L mRNA expression by semiquantitative RT-PCR analysis and found no ADR or HGF/SF alterations in Bcl-X_L mRNA levels in multiple repeat assays.(6) Thus, the alterations in Bcl-X_L protein levels probably occur through translational or posttranslational mechanisms. This finding suggests that some of the protection conferred by HGF/SF may be attributable to alterations in protein processing and metabolism. We had also noted that cell protection required a relatively long preincubation with HGF/SF of $\geq$24 h for some protection and $\geq$48 h for maximal protection (5). This consideration suggests that the ability of HGF/SF to block the reduction of Bcl-X_L protein levels induced by ADR might be attributable to alterations in the expression of genes involved in the processing or metabolism of Bcl-X_L.

Our findings suggest the viability of the cDNA microarray approach, coupled with additional studies to confirm gene expression alterations and functional studies to evaluate the significance of the findings, as a means of identifying novel and interesting genes that may be involved in HGF/SF cell protection pathways. It is likely that some of the genes for which expression was altered by HGF/SF in the setting of DNA damage are not involved in cell survival or apoptosis pathways. Alterations in these gene products may reflect other activities of HGF/SF than promotion of cell survival or may be a passive consequence of cell survival rather than a cause of survival. On the other hand, it is also likely that genes not implicated previously in cell survival or apoptosis mechanisms will be found to play roles in these processes (*e.g.*, *CIP4*).

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Cytogenetics and Cell Genetics

Cell lines from the same cervical carcinoma but with different radiosensitivities exhibit different cDNA microarray patterns of gene expression

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Dedicated to Professor Dr. Ulrich Wolf on the occasion of his retirement.

Abstract. Combining chemotherapy with radiotherapy has improved the cure rate among patients with cancers of the cervix. Although one-half to two-thirds of the patients can be cured by radiation alone, such patients cannot be identified at present and must therefore suffer the burden of chemotherapy. Our long-range goal is to identify those cervical cancers that are radiosensitive and could be cured by radiotherapy alone. The advent of methods that permit the simultaneous analysis of expression patterns of thousands of genes, make it feasible to attempt to identify the molecular events related to radiosensitivity and the associated regulatory pathways. We hypothesize that the sensitivity of tumor cells to ionizing radiation (IR) is determined by the level of expression of specific genes that may be identified with the aid of cDNA microarrays. As the first step in testing this hypothesis, we determined the gene expression differences between two cell lines exhibiting different degrees of radiosensitivity. These were derived from the same tumor prior to treatment from a patient with squamous cell carcinoma of the cervix. The mRNA from these cells was subjected to cDNA analysis on a microarray of 5,776 known genes and ESTs. The expression of 52 genes of the total of 5,776 was elevated (maximum 4.1 fold) in the radioresistant cells as compared to the radiosensitive cells. Ten of the 52 sequences are known genes while 42 are ESTs. Conversely, the expression of 18 genes was elevated in the sensitive cells as compared to the resistant cells. Seven of these 18 are known genes while eleven are ESTs. Among the genes expressed differentially between the resistant and sensitive cells were several known to be associated with response to IR and many more genes and ESTs that had not previously been reported to be related to radiosensitivity. The genes that showed the greatest overexpression in the radioresistant cell line were metal-regulatory transcription factor-1, cytochrome P450 CYP1B1, adenomatosis polyposis coli, translation elongation factor-1, cytochrome-c oxidase, whereas in the sensitive cell line, transcription factor NF-kappa-B, metalloproteinase inhibitor-1 precursor, superoxide dismutase-2, insulin-like growth factor binding protein-3, guanine nucleotide-binding protein and transforming growth factor beta-induced protein were overexpressed.

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Combining chemotherapy with radiotherapy has improved the survival rates of patients with cervical cancers (Keys et al., 1999; Morris et al., 1999; Rose et al., 1999; Whitney et al., 1999). Although one-half to two-thirds of the patients can be cured by radiation therapy alone and do not need chemotherapy, these cannot now be identified so that such patients must unnecessarily suffer the toxicity and the expense of chemother-

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apy. The advent of microarray gene expression technology permits the simultaneous analysis of the levels of expression of thousands of genes. Thus, the study of molecular genetic events that are related to radiosensitivity can be examined. This may also lead to identifying genes and gene regulatory pathways related to the resistance of cells to therapeutic procedure. One of our long-range goals is to use this technology to identify those cancers that are radiosensitive and can thus be cured by radiotherapy alone. Another goal is to identify those cancers that are not controlled by the combined therapy and thus hopefully identify molecular targets for the development of therapeutic strategies.

We hypothesize that the sensitivity of tumor cells to ionizing radiation (IR) is dependent on alterations in the expression of specific genes. As the first step in testing this hypothesis, we determined the differences in the gene expression profiles of two cervical cancer cell lines derived from the same tumor but exhibiting very different degrees of radiosensitivity. We present the results in this report.

Materials and methods

Cell culture

Several cervical cancer cell lines that were derived prior to treatment by punch biopsies from patients with cervical cancers were kindly provided to us by Dr. Richard A. Britten of Cross Cancer Institute, Edmonton, Canada. These were in the fourth to fifth passage. For this report we used one pair of cell lines derived from the same tumor (HT137). These cell lines were cultured in the same way as described by Allalunis-Turner et al. (1991) and Britten et al. (1996). Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum and antibiotics was used. The cells were subcultured every 4–5 days to ensure exponential growth.

Clonogenic cell survival

Following the procedures of Britten et al. (1996) clonogenic survival tests were performed. Briefly, cells were plated in 100-mm petri dishes at known densities and after 4–6 hr the cells were exposed to 2 Gy of radiation using a linear accelerator (Clinac 6-100, Varian oncology systems, Palo Alto CA). After 2 weeks the surviving colonies were stained with crystal violet solution and stained colonies containing more than 50 cells were counted. The surviving fraction (SF) after exposure to 2 Gy for the HT137R cells was thereby determined to be 0.67, and 0.35 for the HT137S cells.

Microarray sample preparation

The cDNA microarray chips and the image scanning programs were developed in the Genome Microarray Facility of the Albert Einstein College of Medicine. The human cDNA microarray chips used in this study each contain 5,776 cDNA sequences representing arbitrarily selected known genes, housekeeping genes and ESTs. The cDNA sample from the radiation sensitive cell line HT137S was labeled with the fluorescent dye, Cy5 (red) and that of the resistant cell line HT137R was labeled with Cy3 (green). A customized ScanAlyse program (Eisen et al., 1998) was used for post-acquisition processing and for database mining functions. The fluorescent signals representing hybridization to each arrayed sequence were analyzed to determine the relative amount of mRNA that hybridized with each sequence in both samples. Full details of the procedure are given on our website: http://sequence.aecom.yu.edu/bioinf/funcgenomic.html.

Synthesis of labeled cDNA probe

One hundred micrograms of total RNA each were isolated from the HT137S and HT137R cell pellets using the Qiagen RNeasy extraction kit. The RNA samples were incubated separately with Oligo dT12–18 at 65°C for annealing of oligo primers. Two mixtures were prepared, one containing first strand buffer, DTT low dNTP mix, RNAsin and the fluorochrome Cy3 for HT137R cells. The second mixture was the same except that Cy5 was used for the HT137S cells. To these mixtures reverse transcriptase (RT, BRL Superscript II) was added and incubated at 42°C for 2 h. Then, to inactivate the RT, the tubes were heated at 94°C. The volume of this mixture was increased to 100 µl by adding buffered RNAse 1 and incubated at 37°C to digest any RNA not converted to cDNA. Then the contents of both the tubes were mixed and passed through Microcon YM 50 retention columns. The probe was collected and the volume adjusted to 6.5 µl.

Table 1. Fifty-two genes and ESTs (out of the total 5,776) whose expression was elevated at least two-fold in the HT137R (Resistant) cells compared with the HT137S (Sensitive) cells

GB Accession number	Description of genes/ESTs
T72724	EST
T80917	EST
R79518	EST
H83358	EST
N42169	EST
N43977	EST
W90242	EST
AA004354	EST
AA004921	EST
AA004570	EST
AA005086	EST
AA010280	EST
AA203495	Metal-regulatory transcription factor-1
H21756	EST
H06460	EST
W02900	Cytochrome P450 CYP1B1, dioxin-inducible
N90485	EST
R00760	EST
R23082	EST
R33908	EST
N28450	EST
H29191	Adenomatosis polyposis coli, alt. Splice-1
T83093	EST
R69208	EST
H82175	EST
N33565	EST
T99685	EST
R31339	EST
R37928	EST
H20450	EST
H40309	EST
N49030	EST
N36501	Phosphodiesterase
N28330	Glycoprotein MUC18. alt. Splice-2
N28369	EST
N78414	EST
T79703	EST
T85390	EST
T86312	EST
T86315	Neurotoxin, eosinophil-derived
T87438	EST
AA190599	Translation elongation factor-1, gamma
H83614	EST
AA028123	EST
H21167	EST
H46937	EST
R83166	EST
R92654	EST
N31224	Glycoprotein MUC18, alt. Splice-3
N99222	EST
H52746	Cytochrome –c oxidase, IV subunit
AA146629	Catenin, alpha 2(E), alt. Splice-1

Array slide preparation

The microarrays on slides were vapor moistened and quickly exposed to 200 mJ total energy in a UV Stratalinker. The slides were moistened again over boiling water and quickly dried on a hot plate. They were then treated with succinic anhydride solution for 15 min, rinsed in 0.1% SDS followed by water, and placed in a 95°C water bath for 3–5 min after which they were dipped into ice-cold ethanol. Excess ethanol was removed from the slides by spinning the rack of slides at 500 rpm.

Fig. 1. A portion of the ScanAlyse picture from the cDNA microarray. The green spots represent genes whose expression is two-fold or greater in the HT137R cells than in the HT137S cells. The red spots represent genes whose expression is two-fold or greater in the HT137S cells. The yellow spots represent genes that are nearly equally expressed in both types of cells. The violet spots are "flagged" spots, i.e. those with dust/specks or similar artifacts. These are flagged to avoid their inclusion in the analysis. Circular customized grids covering each of the 5,776 spots are superimposed on the ScanAlyze spots, to assure the import of the correct color intensity of each spot area into an Excel file for data analysis.

Prehybridization of slide and probe

Prehybridization solution (20 µl, containing formamide, SSPE, SDS, Denhardt's solution and salmon sperm DNA) was placed on the array and covered with a coverglass. The slides were placed in a chamber and prehybridized at 50°C for one hr. Simultaneously the probe was prehybridized at 50°C for 1 h. For prehybridization the 6.5-µl probe was mixed with blocking solution (human cot-1 DNA, SDS, Denhardts and SSPE solution) to bring the volume to 20 µl. It was then heated, centrifuged and incubated as described above. After 1 h both the slides and the probe were ready for hybridization.

Hybridization and washing the slides

The prehybridized probe (20 µl) was dropped on the array region of each slide and a fresh coverslip was placed over the slide. Hybridization was performed overnight at 50°C.

For washing the slides were placed in a glass slide holder containing 1× SSC and 0.1% SDS at room temperature, where the coverslips fell off. The slides were then removed and placed in another slide holder containing 500 ml of 0.2% SSC and 0.1% SDS for 15 min. The slides were then transferred to another chamber, containing 0.2 × SSC for 20 min, after which they were ready for scanning.

Analysis of microarray results

The hybridization signals were scanned with a laser confocal scanner which generates 2-color TIF images. Scans for the two fluorescent probes were normalized to the fluorescence intensity of beta actin and GPDH (Heller et al., 1997) and the ratios of the fluorescence intensities of all the spots was determined. Intensities for each spot in each channel were calculated after subtraction of the background. Background "noise" was reduced by using a 2-standard deviation cutoff on all expression values in order to identify only those genes with significantly different expression (Chen et al., 1997; Eisen et al., 1998; Amundson et al., 1999; Duggen et al., 1999; Lee et al., 1999; Pollack et al., 1999). Red spots represent genes whose expression in the HT137S cells is at least double that of the HT137R cells. Green spots represent those genes whose expression was double in the HT137R cells as compared to the HT137S cells, whereas yellow spots represent genes whose expression was similar in both the cell lines (Fig. 1). Genes and ESTs had to be expressed at similar fluorescent intensity ratios in at least two microarray hybridizations to be included in the analysis.

Results

The expression of 52 genes (0.9%) out of the total 5,776 was elevated (2–4.1 fold) in the HT137R cells as compared to the HT137S cells (Table 1). Ten of these 52 are known genes, while 42 are ESTs. Conversely, the expression of 18 genes was elevated 2–2.9 fold in the HT137S cells compared with the HT137R cells (Table 2). Seven of these 18 are known genes while 11 are ESTs. The genes that showed the greatest overex-

Table 2. Eighteen genes (out of the total 5,776) whose expression was elevated at least two-fold, in the HT137S (Sensitive) cells compared with the HT137R (Resistant) cells

GB Accession number	Description of genes/ESTs
R86053	Transcription factor NF-kappa-B
R97630	Alcohol dehydrogenase-1, class 1, alpha polypeptide
N67954	EST
R78823	EST
AA001324	EST
T99143	EST
N57354	EST
AA143155	Superoxide dismutase 2, mitochondrial, alt. Splice-1
R75975	Monocyte chemotactic protein -1
R25247	EST
R38114	EST
R80595	EST
N31417	Insulin-like growth factor binding protein 3
N28758	EST
R78657	Guanine nucleotide-binding protein HM89
N42864	EST
AA002125	EST
AA037281	Transforming growth factor beta-induced protein

pression in the radioresistant cell line were metal-regulatory transcription factor-1, cytochrome P450 CYP1B1, adenomatosis polyposis coli, translation elongation factor-1 and cytochrome-c oxidase, whereas in the sensitive cell line, transcription factor NF-kappa-B, metalloproteinase inhibitor-1 precursor, superoxide dismutase-2, insulin-like growth factor-binding protein-3, guanine nucleotide-binding protein and transforming growth factor beta-induced protein were overexpressed.

Discussion

It is clear from the results that cell lines from the same tumor but with different radiosensitivities exhibit different patterns of gene expression. Having determined that such differences exist the next question to be answered is to what extent are these gene expression alterations related to radiosensitivity. We hope to be able to answer this question by examining a larger series of similar cell lines and also radiosensitive and radioresistant primary cervical carcinomas. If the same specific genes have altered expression in many different cases then this would be evidence that they are related to the cell's response to radiation. It is encouraging that among the genes expressed differentially between the resistant and sensitive cells in this study were several that are known to be associated with the cell's response to IR. These are: transcription factor NF-kappa-B, superoxide dismutase-2, insulin-like growth factor-binding protein-3, guanine nucleotide-binding protein, and transforming growth factor beta-induced protein (Arnold et al., 1999; Kawai et al., 1999; Epperly et al., 2000; Kuninaka et al., 2000; Williams et al., 2000). As noted there were also a number of genes and ESTs which have previously not been reported to be related to radiosensitivity (Tables 1 and 2). Their importance in conferring the radioresponse phenotype to a cell will clearly require much more extensive studies, particularly because the phenomenon appears to be fairly complex involving several genes and gene pathways. As an example, IR-induced patterns of gene expression may vary according to the cellular context as demonstrated by Amundson et al. (1999) who studied IR-induced gene expression in human myeloid ML-1 cells using a microarray consisting of 1,238 gene sequences. They found that 48 sequences (including 30 not previously identified as IR-responsive) were significantly influenced by IR. Induction by IR of a subset of these genes was examined in a panel of 12 human cell lines, and it was observed that the responses varied widely in cells from different tissues of origin and different genetic backgrounds (Amundson et al., 1999).

Like many other investigators we have regarded as significant only those genes whose expression was altered by at least a factor of two. However, we recognize that this cutoff point is arbitrary and that there may be important genes involved whose expression was altered by less than a factor of two. Another limitation of this study is that the microarray utilized consisted of only 5,776 arbitrarily selected known genes, housekeeping genes, and ESTs. This limitation can now be overcome since microarrays with much larger numbers of genes are now available. In addition, customized arrays are becoming available with genes known to, or suspected of, participating in the process under study. An example of the successful application of the latter approach is the recent demonstration with a "lymphochip" that large-cell lymphomas responding well to CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy could be distinguished from those responding poorly (Alizadeh et al., 2000).

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Differential gene expression associated with tumorigenicity of cultured green turtle fibropapilloma-derived fibroblasts

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Abstract Fibroblast cell lines derived from normal skin and experimentally induced fibropapillomas of green turtles (*Chelonia mydas*), were propagated in vitro and tested for tumorigenicity in immunodeficient mice. Differential display RT-PCR was used to identify differences in messenger RNA expression between normal and tumorigenic fibropapillomatosis (FP)-derived fibroblasts from the same individual. Four unique products that were apparently overexpresed in FP and three that were apparently underexpressed were cloned and sequenced. Differential expression was confirmed for three products by Northern blotting. Two overexpressed products showed extensive sequence matches to the known mammalian cellular genes, beta-hexosaminidase and chain termination factor. The product that was underexpressed in FP showed homology with mammalian thrombospondin, a known tumor-suppressor gene and an inhibitor of angiogenesis. All of the partial gene sequences identified are novel and will require full length cDNA sequencing to further analyze their identities. These results, however, provide the foundation for further investigation to determine the role of each of these gene products in FP pathogenesis and cellular transformation. The potential for some of these products to serve as biomarkers for FP is discussed.

1. Introduction

The green turtle, *Chelonia mydas*, is an endangered species. In the past two decades, populations of green turtles around the world have been affected by an increasing prevalence of fibropapillomatosis (FP), a disease that is characterized by multiple cutaneous and occasional visceral fibromas or fibrosarcomas [1]. This disease kills a considerable proportion of severely affected turtles and in the rest it increases the susceptibility to other mortality factors such as predation.

A major histologic feature of FP in all tissues is the proliferation of stromal fibroblasts, which suggests that fibroblasts are the cells that have undergone pathological changes [2]. Transmission experiments have implicated a viral agent as the cause of FP [3,4], but the mechanism is unknown. Possible mechanisms include either direct transformation of infected fibroblasts (neoplasia), or paracrine stimulation of uninfected fibroblasts (hyperplasia) by another cell type that is infected or transformed by the agent. These tumor fibroblasts are well differentiated and have normal cytologic features and are morphologically indistinguishable from normal dermal fibroblasts and have similar growth patterns and serum dependence in vitro [5]. This has made comparative studies of these cells difficult, because to elucidate the molecular basis of FP fibroblast proliferation, these cells should be differentiated from normal fibroblasts. However, we have shown that FP-derived fibroblasts are tumorigenic whereas normal dermal fibroblasts are not, using an immunodeficient mouse model [5].

The purpose of this study was to begin to elucidate the molecular mechanisms of FP pathogenesis by searching for differences in gene expression between closely matched sets of tumorigenic FP-derived and non-tumorigenic normal dermal fibroblast cell lines using differential message display analysis [6]. This is a sensitive technique to identify both novel viral genes that may be differentially expressed in infected or transformed cells and also host genes whose expression is altered by infection with the FP agent or any other transforming events.

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2. Materials and methods

2.1. Cell lines

Pairs of matched early passage tumor and normal skin-derived fibroblast lines derived from green turtles with experimentally induced FP were propagated at 30°C in a 5% CO_2 atmosphere in Dulbecco Modified Eagle Media (D-MEM)/F12 supplemented with 10% FBS (GIBCO, Grand Island, NY, USA) as described previously [5]. Cultures were expanded to approximately 6×10^7 cells. Cells were grown to confluence and then harvested with 0.25% trypsin-1 mM EDTA in Hanks' Balanced Salt Solution (HBSS). The cells were washed in HBSS and approximately 5×10^7 cells were used for DNA and RNA extractions, and 1–5×10^6 cells were used in tumorigenicity assays to confirm their phenotype. The remaining cells were cryopreserved for further investigation.

2.2. In vivo tumorigenicity

Tumorigenic potential of both tumor and normal skin-derived fibroblast cell lines were evaluated using the immunodeficient Rag-2 −/− or C.B17-*scid/scid* mice, as described in Herbst et al. [5]. Aliquots of 1–5×10^6 cells suspended in 100 μl PBS were injected into the margin in the pinna and the mice were observed weekly for at least 4 months for evidence of tumor development.

2.3. DNA extraction

DNA was prepared from approximately 1×10^7 cells using standard proteinase K digestion followed by phenol: chloroform:isoamyl alcohol extraction and ethanol precipitation [7].

2.4. RNA extraction

Total RNA was extracted from 3×10^7 cell using a RNA extraction kit (Stratagene, La Jolla, CA, USA; # 200345) as per manufacturer's protocol.

2.5. Differential message display RT-PCR

Briefly, the total RNA preparations were treated with RNAse-free DNAse to remove possible chromosomal DNA contamination. The cDNA was synthesized from the total RNA samples by reverse transcription using 4 sets of degenerate anchored (3′) primers (T12MN) where M is G, A, or C and N is G, A, T, or C. With 12 possible combinations of the last 2 bases, each primer recognized 1/12th of the total mRNA population. Partial cDNA sequences were amplified using 5′ end primers, corresponding 3′ end primers, and ^{35}S labeled dATP such that 50–100 cDNAs were amplified. The 5′ primers were arbitrary decamers allowing annealing positions to be randomly distributed in distance from the polyA tail. The 5′ primers were designed to maximally randomize the 3′ end with a fixed 5′ end. Following amplification, short 100–500 bp cDNA sequences were separated on polyacrylamide sequencing gels. The products from tumorigenic and normal cells were run on adjacent lanes, allowing side-by-side comparison of the mRNA expression pattern of tumorigenic versus normal skin fibroblasts. Bands that were present in one cell line but absent in the other were cut from the gel, cloned into pGEM easy vector systems (Promega Corporation; Cat# TM042) and sequenced manually.

2.6. Northern blotting

Cloned DD-RT-PCR products were ^{32}P-dTTP labeled and used as probes on Northern blots to verify differential expression. Total RNA samples (30 μg per lane) from matched pairs of cell lines (FP and normal) were run on agarose gels and blotted onto nitrocellulose membranes. Probes were hybridized for 72 h at 42°C and washed in 2× SSC [7].

3. Results

The cultured tumor-derived fibroblasts were morphologically indistinguishable from cultured normal fibroblasts under light microscopy as observed previously [5]. The FP-derived fibroblast lines, however, were tumorigenic when injected into in the ears of *Rag2* −/− or *scid/scid* mice whereas the normal fibroblasts did not develop tumors.

DD-RT PCR yielded several cDNA segments that appeared to be either overexpressed or underexpressed in vitro in tumorigenic FP fibroblasts compared to normal fibroblasts (Fig. 1). Of these, four unique overexpressed products (ranging in size from 189 to 412 bp) and three underexpressed products (193–401 bp) were cloned and sequenced.

3.1. FP overexpressed transcripts

Positive (sense) strand homologies to expressed sequence tags (ESTs) and short coding regions were found for all of the four products, however, extensive matches of the full length product sequence to 3′ cDNA of known genes were found for only two of these products (LHHCM4-5 and LHHCM8-3). Both of these were confirmed by Northern blots to be overexpressed in tumor compared to normal (Fig. 2). The other two clones (LHHCM2-2 and LHHCM7-4) didn't show any homology in the GeneBank and surprisingly their differential expression could not be detected in the Northern blotting experiments in either tumor or normal fibroblast RNA under the conditions used. Repetition of these experiments with excess amounts of RNA will determine if these transcripts are actually low in copy number.

The first 31 nucleotides of product LHHCM8-3 (386 bp) had sense strand homology to the 3′ end of pig and human beta-hexosaminidase (X92379.1 and HUMHEXB, respectively), and the putative amino acid sequence was homologous to the 3 terminus of beta-hexosaminidase transcript of the pig (Q29548) and human (P06865). A putative amino acid region of 153 bp long had 38/51 (74%) amino acid matches to the 3′ terminus of the pig hexosaminidase mRNA. The remaining portion of product LHH8-3 was a non-peptide sequence.

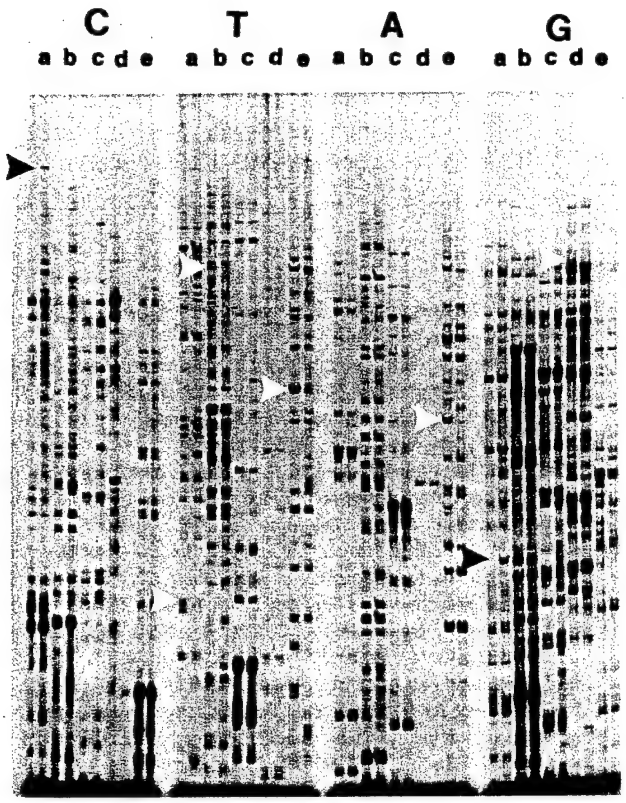

Fig. 1. Gene expression profiles of genes by differential message display studies using total RNA, isolated from a pair of matched tumor and normal skin derived fibroblast cell lines cultured from a turtle with experimentally induced FP. Columns C, T, A, and G represent each of the 3′ primers ($T_{12}CN$ where N = C, T, A, and G, respectively). Subcolumns a, b, c, d, and e represent individual 5′ primers (arbitrary decamers). For each of the 20 primer pair combinations, products of normal fibroblasts (left) are displayed along side those from tumor fibroblasts (right). Differentially expressed gene sequences (open and closed arrowheads) were cloned and sequenced for further characterization.

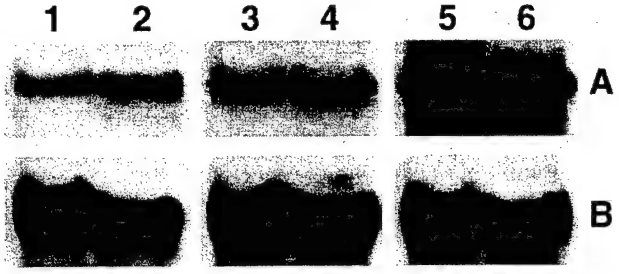

Fig. 2. Northern blotting of total RNA of normal (lanes 1, 3 and 5) and tumor (lanes 2, 4 and 6) cells using three probes derived from the differential product sequences. Lanes 2 and 4 show over-expression of two sequences (LHH4-5 and LHH8-3) and lane 6 shows under-expression of sequence LH10-5 in the tumor samples. The 18s RNA probe (B) was used to confirm equal loading of RNA samples.

Product LHHCM4-5 (412 bp) was homologous over its entire length to the 3′ terminus of eukaryotic peptide (AB029089) and hamster (MAC114, MAC111). Comparison of LHHCM 4-5 with the full-length human cDNA (HSHCGVII) revealed 4 short gaps of 3, 19, 2, and 23 bp in the sequence alignments and was 88% identical (207/235bp) if the gaps are not considered. Interestingly the putative 3′ end of LHHCM 4-5 contained a 130 bp sequence, which was unique. The product is not in the amino acid coding part of the transcript.

3.2. FP underexpressed transcripts

Three RT-PCR products that appeared to be relatively underexpressed in FP fibroblasts were also successfully cloned and sequenced. Only one of these clones, LHHCM10-5 (321 bp) was confirmed to be underexpressed in FP by Northern blotting (Fig. 2). This sequence had only limited homology to known mammalian sequences. The longest of the sense strand matches was human and bovine thrombospondin-1 mRNA, which contained a 96 bp sequence that was 83% identical. Sequence LHHCM5-3 (359 bp) had partial sequence homology to chicken delta EF1, a transcriptional repressor (58% base pair identity over 90 bp) and to a region containing human HLA class II gene sequence (68% base pair identity with two gaps of 77 bp and 4 bp, respectively, over 140 bp). This transcript was not detected by Northern blotting in either FP or normal fibroblast RNA under the conditions used, suggesting its low copy number. Product LHHCM1-4 (401 bp) hybridized to RNA from both cell types but was not differentially expressed. This sequence did not have any obvious matches to known genes in the databases.

4. Discussion

The availability of matched green turtle FP-derived and normal fibroblast lines and a model system in which to monitor the tumorigenic phenotype made it possible to investigate the molecular basis of fibroblast proliferation in green turtle fibropapillomatosis.

Comparison of gene expression in matched tumorigenic FP-derived versus non-tumorigenic normal skin-derived fibroblasts from individual green turtles with experimentally induced FP yielded several cDNA products by RT-PCR. Two of these were confirmed to be overexpressed in tumor fibroblast RNA and one was confirmed to be underexpressed by Northern blotting. The fact that some of the remaining products could not be detected in Northern blots of either cell type may indicate that these transcripts were very low in copy number. The total RNA was extracted from mass cultured fibroblasts, which could contain an undetermined proportion of tumor cells mixed with normal cells. Therefore, differences in gene expression that were detected by Northern blotting are probably robust differences and more subtle differences will require cloned cell lines.

Very few turtle or reptilian gene sequences have been available in the gene sequence databases. Consequently, all of the partial cDNA sequences found in this study are novel

and cannot be assigned with certainty to their putative corresponding mammalian homologue. In addition, some of the over-expressed products may represent viral gene transcripts from the FP transmissible agent, if it latently infects fibroblasts. Preliminary data from these cell lines suggest that FP fibroblasts are non-productively infected with a green turtle FP-associated herpesvirus, a candidate for the FP etiologic agent [8], so it is possible that some of these sequences could also belong to the herpesvirus genome. For each product, the full-length cDNA must be sequenced and then studied further to characterize putative gene function and to determine whether a corresponding polypeptide is expressed. Extensive additional gene sequence information for green turtles and FP-associated turtle viruses will also be needed before these products can be properly identified and before it can be determined if these products represent normal transcripts whose expression level has changed or aberrant transcripts resulting from gene mutation, activation of pseudogene expression, or abnormal RNA processing.

These results provide a foundation for developing hypotheses about the pathogenesis of fibroblast transformation in FP. For example, the role of polypeptide chain releasing factor or eukaryotic RF1, the putative identity for product LHHCM4-5, in neoplasia or viral infection deserves further investigation. This protein is responsible for chain termination at all 4 stop codons [9]. Overexpression of an aberrant form of this protein, argueably may disrupt an important checkpoint in preventing the translation of abnormal mRNA transcripts, which could enhance cancer progression [10,11].

Product LHH10-5, which is underexpressed in FP fibroblasts, is a putative mammalian homologue of thrombospondin. Thrombospondin is an inhibitor of angiogenesis and a known tumor suppressor [12–14]. Its expression is decreased in a number of neoplastic diseases and in cells infected with human cytomegalovirus, a herpesvirus, as well [15].

The putative identity of LHHCM sequence 8-3, is beta hexosaminidase based on base pair and amino acid homologies to the mammalian gene. Isozymes of beta-hexosaminidase have been shown to be overexpressed in other types of neoplasia [16–21] and increased levels have been detected in the serum of virus infected humans [22]. Thus over expression of this gene is expected in FP. Therefore, isozymes of this protein may serve as useful serum markers for turtles with cryptic FP, such as visceral tumors, or systemic virus infection.

As stated, further elucidation of the role of these genes in FP tumorigenesis or FP-virus infection will require, identification of their full length cDNAs. To establish their respective roles in tumorigenesis will require both in vitro and in vivo studies, such as transfection and overexpression of transcripts in normal fibroblasts to determine if they become tumorigenic. Fortunately, a model system has been developed and matched cell lines are available in our laboratory, which we believe would allow further elucidation of FP in turtles.

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Annexure VII

Abstracts and Presentations

AMERICAN ASSOCIATION FOR CANCER RESEARCH

90th

APRIL 10-14, 1999 PHILADELPHIA, PA

Volume 40 March 1999

In cooperation with

FOX CHASE CANCER CENTER

#2845 Search for novel molecular markers to identify patients with breast cancer at high risk for developing metastases using representational difference analysis. Achary, P.M., Mukherjee, B., Khaimov, R., Fan, Z., Mahadevia, P.S., and Vikram, B. *Department of Radiation Oncology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY 10461.*

Using single cell microdissection, we isolated normal cells and cancer cells from a metastatic lymph node from a woman with breast cancer. Representational difference analysis (RDA) was performed to isolate genomic DNA sequences that had undergone alteration during progression from normal to metastatic phenotype. RDA has yielded 11 differential sequences. Dot/ Southern blot analyses have confirmed that these sequences were present in normal but lost from the metastatic cell DNA. The preliminary characterization of one of the novel sequences, M-41, has been undertaken in another four patients with invasive ductal primary breast cancers with lymph node metastases. From each patient's specimens, ~1000 cells each were microdissected from the normal tissue, the primary tumor and the metastasis. Genomic DNA was isolated from each of these 12 samples, and amplified by a method recently standardized in our laboratory. In each of the 4 patients, we have observed strong hybridization signals from the normal and primary tumor DNA, and only faint signals from the metastatic DNA, suggesting a potential metastasis-specific genetic marker. In addition to repeating these studies, we are screening few more patients' specimens.

SCIENTIFIC PROCEEDINGS

91st Annual Meeting of the American Association for Cancer Research

San Francisco, CA
April 1–5, 2000

Abstracts are numbered from 1 through 5595; however, several numbers may be omitted in the sequence. Abstracts were either typeset from a paper copy or received directly from the authors by electronic submission. Every effort has been made to reproduce the content of the abstracts according to the paper copy submitted, except in certain instances where changes were made to comply with AACR style. AACR does not assume any responsibility for proofreading or correcting any scientific, grammatical, or typographical errors, nor does AACR assume responsibility for errors in the conversion of customized software, newly released software, or special characters. No responsibility is assumed by the AACR, publisher and copyright owner of the Proceedings; by the Keck School of Medicine of the University of Southern California; or by the meeting organizers for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or for any use or operation of any methods, products, instructions, or ideas contained in the material herein. Independent verification of diagnoses and drug dosages should be made by readers or users of this information.

Overview and Objectives of the Annual Meeting

The purpose of the AACR Annual Meeting is to present the most timely and significant research results in all of the scientific disciplines relevant to cancer. All scientists studying the causes, diagnosis, treatment, and prevention of cancer will benefit from attending this meeting. After participating in this continuing education activity, the participant should be better able to understand the latest research findings in all areas of cancer research; implement these findings in diagnosis, treatment, and prevention of cancer; and use these findings to make further progress in research efforts.

Joint Sponsorship Accreditation Statement

This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education (ACCME) through the joint sponsorship of the Keck School of Medicine of the University of Southern California and the American Association for Cancer Research. The Keck School of Medicine of the University of Southern California is accredited by the ACCME to provide continuing medical education for physicians.

The Keck School of Medicine of the University of Southern California designates this educational activity for a maximum of 48 hours in Category 1 credit towards the AMA Physician's Recognition Award. Each physician should claim only those hours of credit that he/she actually spent in the educational activity.

Physicians who wish to receive Category 1 credit should obtain an attendance form from the CME booth located in the Moscone Center near the registration area.

Disclosure Statement for Continuing Medical Education Faculty

The American Association for Cancer Research (AACR) and the Keck School of Medicine of the University of Southern California are committed to ensuring the integrity of their scientific, educational, and research programs and therefore require disclosure of any financial or other interests which might be construed as resulting in an actual, potential, or apparent conflict. The existence of financial interests or other relationships of a commercial nature is not regarded by the AACR or USC as creating a presumption of impropriety. Rather, this policy represents recognition of the many factors that can influence judgments about research data and a desire to make as much information as possible available to those reviewing the data.

All faculty participating in an educational activity are expected to disclose to the activity audience any significant financial interests or other relationships (1) with the manufacturer(s) of any commercial products and/or providers of commercial services discussed in an educational presentation and (2) with commercial supporters of the activity. (Significant financial interest or other relationship can include such things as grants or research support, employee, consultant, major stockholder, member of speakers bureau, etc.) The intent of this disclosure is not to prevent a speaker with a significant financial or other relationship from making a presentation, but rather to provide listeners with information on which they can make their own judgments. It remains for the audience to determine whether the speaker's interests or relationships may influence the presentation with regard to exposition or conclusion.

A summary of the disclosure information provided by Annual Meeting presenters may be found on pages 897-905 of this *Proceedings*.

Next Annual Meeting: March 24 – 28, 2001, New Orleans, LA

#2742 CDNA MICROARRAY GENE EXPRESSION PROFILE IN A HIGHLY METASTATIC BREAST CARCINOMA CELL LINE. Rafik Khaimov, Eugene Gross, Jiajian Liu, Bhadrasain Vikram, and Mohan P Achary, *Albert Einstein Coll Of Medicine, Bronx, NY*

To identify differential expression of genes associated with breast metastasis, we analyzed low metastatic (HI-177) and highly metastatic (C-100) cell lines, derived from the same source by cDNA microarray. The hypothesis is that genetic alterations leading to the underexpression and overexpression of specific genes are responsible for the ability of a breast carcinoma cell to become low or highly metastatic. We used the microarray chips and the image scanning programs, recently developed in the Genome Microarray Facility of Albert Einstein College of Medicine. Fluorescent cDNA probes for hybridization were derived from HI-177 (Cy3) and C-100 (Cy5) cell lines and, to determine the relative abundance in the two samples of mRNAs corresponding to each gene, the signals representing hybridization to each arrayed gene were analyzed using the ScanAlyze2 software. Even though the origin of these cell lines was the same, we found overexpression of 53 known genes and 66 ESTs and underexpression of 1 known gene and 4 ESTs in the highly metastatic cell line. Northern blots are underway to verify the expression levels of overexpressed genes (e.g. tyrosine kinase, superoxide dismutase, thymidylate synthase) and an underexpressed gene (hsp28) with that of microarray results. If differential expression is confirmed, the genes will be further characterized to determine their role in breast metastasis.

Building Bridges

CUNY CONFERENCE IN SCIENCE & ENGINEERING 2001

Keynote Speaker

Dr. Roosevelt Y. Johnson

National Science Foundation

February 23, 2001

Recital Hall (The Graduate Center)

- Panel featuring scientists in academia and industry.
- Presentations highlighting the work of students in CUNY's MAGNET, AGEP, BRIDGES, PIPELINE, MARC, AMP, MBRS, and CASI programs.
- Student poster sessions.

TO IDENTIFY DIFFERENTIAL EXPRESSION OF GENES ASSOCIATED WITH BREAST METASTASIS

Lilianette Rovira and Mohan Achary
Albert Einstein College of Medicine

The clinical outcome is generally positive for patients with node-negative breast carcinoma who have been treated with surgery or surgery and radiation therapy. IThe long-term objective is to determine the patterns of gene expression specific to each group of these tumors by cDNA microarray, and to analyze them according to treatment and clinical outcome. The hypothesis is that these 2 groups of tumors have distinct patterns of gene expression, which determines the therapeutic response. This may help design improved therapeutic approaches especially for group II tumors and spare the group I patients, the expense and toxicity of chemotherapy. The objective of the current project is to identify cDNA microarray gene expression profile in a highly metastatic human breast carcinoma cell line involved in breast metastasis. We have isolated total RNA from these cell lines and labeled them with Cy3/Cy5 and hybridized to chips containing ~9000 human gene sequences.

THYMIC NURSE CELLS AND MACROPHAGES MAY FUNCTION IN CONCERT DURING MHC RESTRICTION.

Michael Samms, F. Emanus, M. Martinez, S. Fousse, M. Pezzano and J. Guyden. The City College of New York, CUNY

Thymic nurse cells (TNCs) are cortical epithelial cells primarily found in the thymus. TNCs have been shown to contain as many as fifty thymocytes within their cytoplasm and express high levels of MHC class II on their cell surfaces. Thus, TNCs have been implicated in MHC restriction. To further elucidate TNC involvement in T cell selection our laboratory has developed thymic nurse cell lines (tsTNC-1) that selectively bind and internalize immature abTCRloCD4+CD8+ thymocytes in vitro. A subset of the population released from the TNC interaction was shown to mature to the abTCRhiCD69hi stage of development while thymocytes that remained within the TNC cytoplasm died through the process of apoptosis. Here, we show the presence of both apoptotic and non-apoptotic thymocytes within the cytoplasm of freshly isolated TNCs as well as tsTNC-1 cells in culture. More recently, we have detected macrophages within freshly isolated TNCs. This finding supports one earlier report which showed TNCs to harbor macrophages. However, when peritoneal macrophages were pre-treaded with CFDA SE invitro and injected into the peritonal cavity of C57B/6 mice, we detected peritoneal macrophages within the thymus and more specifically within TNCs after 2 hours. We have observed the movement of macrophages into and out of TNCs in culture. Taken together, the data suggest that macrophage work in concert with TNCs during the process of thymocyte selection.

APOPOTOSIS, CELL'S DEATH

Leonardo Santana
Bronx Community College, CUNY

Apoptosis is a programmed cell's death by their biochemical mechanism. Apoptosis play an important role during development on vertebrate animals, by regenerating the cells involve in differentiation. However, Apoptosis has linked with different neurodegenerative diseases, such as Alzheimer and Schizophrenia, etc. Kainic acid, neurotoxin use as a nurobiological tool to induce apoptosis, was injected to rats 10 to 12 weeks old. Brain tissue from four different regions (olfactory bulbs, frontal lobes, hippocampus and occipital) of the brain were collected and tested for positive apoptotic cells. Using a method call (TUNEL), we were able to detect DNA fragmentation; one of the characteristic of apoptotic cell death. The grater concentration of positive cell was detected in the olfactory bulbs and frontal section of the brain with a reduction of them in the hippocampus, and no activity as detected in the occipital region. Because some of the symptoms in-patients with neurodegenerative diseases have been attributed to absence of oxygen in brain cells, this study my have important implications in the understanding of such diseases, by the measures of oxidative markers.

THE FIFTH ANNUAL

EINSTEIN POSTDOCTORAL SYMPOSIUM

Poster Session ABSTRACTS

December 4, 2001

Albert Einstein College of Medicine
of Yeshiva University

Belfer Institute for Advanced Biomedical Studies

Characterization of Metastasis Associated Gene Sequences in Breast Carcinoma

H. Zhao, Z. Fan, L. Herbst[1], H. P. Klinger[2], B. Vikram and P. Mohan R. Achary
Metastasis Laboratory, Departments of Radiation Oncology, Pathology[1], Molecular Genetics[2], Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY.

About 13% mammary carcinoma patients with negative lymph nodes, even though treated with surgery or surgery and radiation therapy, are still prone to developing metastasis. The objective of this project is to construct a panel of molecular genetic markers for detecting those 13% patients so that they could be treated more aggressively. This is also beneficial to the remaining 87% patients who are not likely to metastasize. These women could then be treated more conservatively, sparing them the considerable physical, mental and financial costs of the treatment and with great ease of mind. The long-term goal of this study is to use these markers for better prognosis as well as the basis for the development of novel therapies in future.

The hypothesis to be tested is that in order to achieve the metastatic state, primary mammary carcinoma cells must acquire genetic changes in addition to those that led to transformation. This may involve the loss of function of metastasis suppressor genes or the activation of metastasis promoting genes such as oncogenes. The existence of both types of genes have been reported in several tumor types, but many more are very likely to exist, and much remains to be learned about the possible roles of such genes in mammary carcinoma. A subtractive DNA hybridization technique, Representational Difference Analysis (RDA) was used to compare the DNA of cells from archival normal tissue or primary ductal tumor with that of the metastatic lymph node of the same patient to isolate those sequences that were lost in the course of tumor metastasis. The tumor and metastatic cells were recovered by laser capture microdissection.

We have isolated 11 candidate metastasis associated gene sequences (MAGS) that were found to be lost in metastatic cells. When screened on normal, primary and metastatic cell DNA samples from 5 breast carcinoma patients one of them (MAGS-XI) was found to be lost in the metastatic cells of 3 out of 5 patients and another sequence (MAGS-IX) in 2 out of 3 patients indicating their involvement in breast metastasis. Presently we are isolating partial or full-lengths of these MAGS using inverse PCR method in order to use these sequences as fluorescence *in situ* hybridization (FISH) probes to screen large number of patient samples.

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Volume 43 • March 2002

In joint sponsorship with the Keck School of Medicine of the University of Southern California

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Proceedings

✓ **#220 A candidate metastasis associated genetic marker for ductal mammary carcinoma.** Hui Zhao, Zuoheng Fan, Lawrence Herbst, Dwayne Breining, Joan G. Jones, Panna S. Mahadevia, Harold P. Klinger, Bhadrasain Vikram, and Mohanrao P. Achary. *Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY, and Albert Einstein College of Medicine and Montefiore Medical Center,.*

Metastasis is responsible for most deaths from breast cancer. The objective of this project is to identify a panel of molecular genetic markers for detecting those 13% of mammary carcinoma patients with negative lymph nodes that are prone to developing metastasis even after surgery, or surgery combined with radiation and/or chemotherapy. This would also benefit the remaining 87% patients, who could be treated more conservatively, sparing them the physical, mental and financial costs of the radical treatment. The long-term goal of this study is not only to find markers for prognosis but also to identify metastasis associated genes. Such knowledge could guide the development of improved therapies. The working hypothesis is that in order to achieve the metastatic state, primary mammary carcinoma cells must acquire genetic alterations in addition to those that led to transformation. In order to isolate genes associated with the suppression of metastasis (whose loss would increase the metastatic potential of the tumor), Representational Difference Analysis (RDA) was used to compare the DNA of cells from archival normal tissue or primary ductal tumors with that of the corresponding metastatic lymph node. The primary tumor and metastatic cells were recovered by laser capture microdissection and DNA samples extracted from those cells were used for RDA and for Southern blotting and PCR methods to screen metastasis associated gene sequences (MAGS). Eleven candidate MAGS were recovered that were apparently lost in metastatic cells. One of these, MAGS-IX when used to screen normal, primary and metastatic cell DNA from 3 additional breast carcinomas, was found to be lost in metastatic cell DNA of two of these samples. Thus in 3 out of 4 cases (including the original case used for RDA) MAGS-IX was apparently lost from those primary tumor cells that became metastatic. This makes MAGs-IX a potential candidate for being a metastasis suppressor gene. RH mapping localized MAGS-IX to a 21cR interval between markers, D105539 and D10S549, on human chromosome 10, band q21.1. Homology searches revealed it to have 94% sequence identity to a clone on human chromosome 10 (AC022541) but not to any other known gene sequence, suggesting

that this is a novel MAGS. Additional archival cases are being screened to determine if MAGS IX is a reliable marker for identifying breast cancers that are prone to becoming metastatic.

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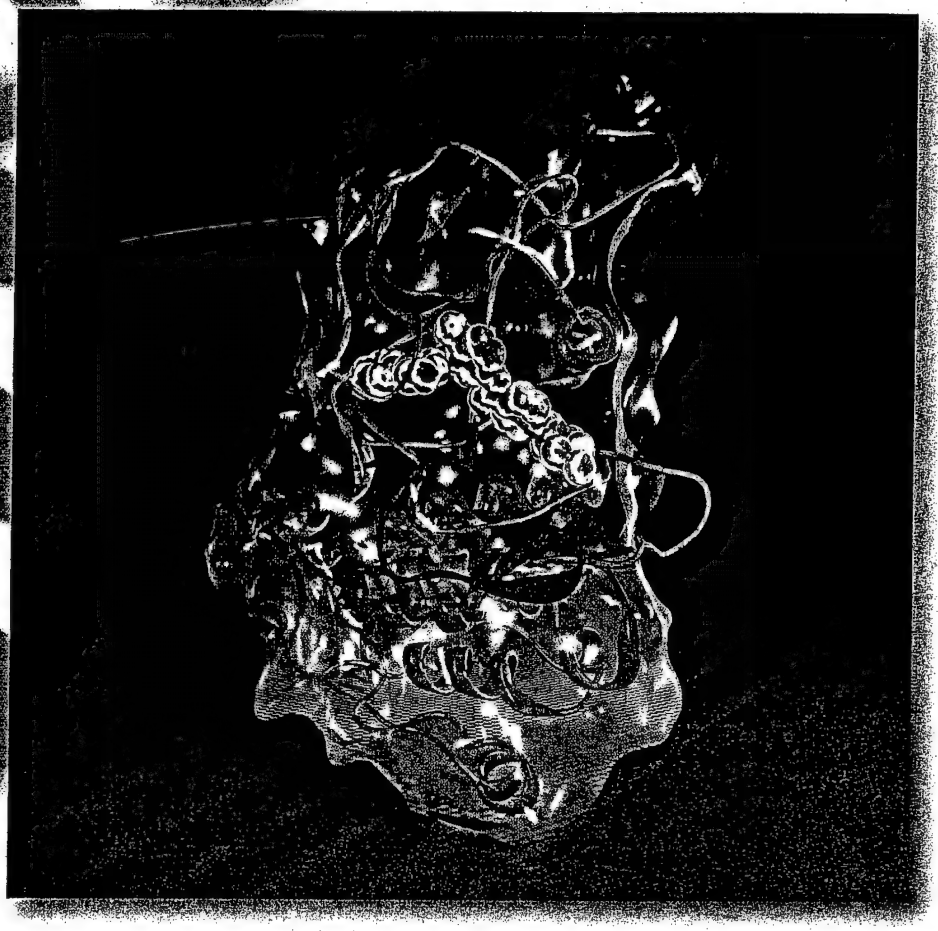


94th Annual Meeting 2003

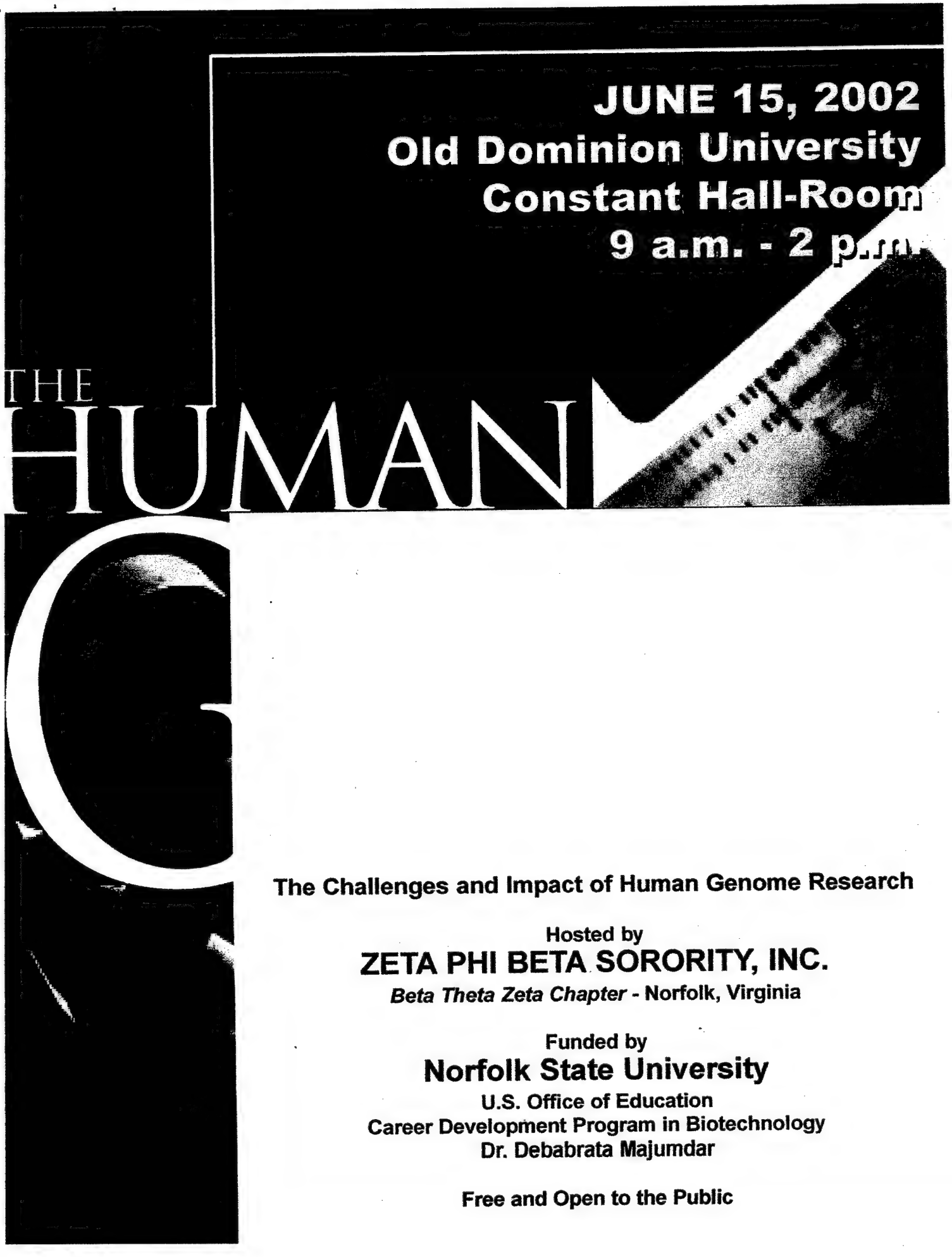
April 5-9, 2003 • Metro Toronto Convention Centre
Toronto, Ontario, Canada

Proceedings

Volume 44
March 2003

#189 Genes and pathways associated with nm23 gene mediated metastasis inhibition in breast carcinoma cells. Hui Zhao, Meena Jhanwar-Uniyal, Vivek Anand, Ilya Kuperschmidt, Gregory Khitrov, Lawrence Herbst, and Mohanrao P. Achary. *Albert Einstein College of Medicine, Bronx, NY, American Health Foundation Cancer Center, Valhalla, NY, I. M. Sechenov Moscow Medical Academy, Moscow, Russia, Silicon Genetics, Redwood City, CA, and Gene Array Resource Center, New York, NY.*

Metastasis of various malignancies has been shown to be inversely related to the abundance of nm23 protein expression. However the downstream pathways involved in nm23 mediated suppression of metastasis have not been elucidated. In the present investigation, we took advantage of cDNA microarrays to identify novel genes, pathways and targets for gene therapy in the nm23 mediated spontaneous breast metastasis. Microarray experiments were performed in two cell lines, namely: C-100 (only vector transfected; highly metastatic) and H1-177 (nm23 transfected; low metastatic), derived from human mammary carcinoma cell line, MDA-MB-435. The analysis of data from 3 independent experiments using GeneSpring software revealed significant as well as consistent alterations in the expression (up and down regulation) of 2,158 genes in a total of 20,000 genes between high and low metastatic cells. The expression of 197 genes (0.98%) was elevated more than 1.7 fold in the low metastatic cells as compared to the highly metastatic cells. Of the 197 sequences, 133 (0.67%) are known genes while 64 (0.32%) are ESTs. Conversely, the expression of 1961 genes (9.8%) was down regulated (< 0.6 fold) in the low metastatic cells or elevated (> 1.7 fold) in the highly metastatic cells. Of the 1961 sequences 811 (4.05%) are known genes while 1150 (5.75%) are ESTs. These genes were grouped into 6 functional categories, namely invasion and metastasis, apoptosis and senescence, signal transduction (molecules and transcription factors), cell cycle and repair, adhesion, and angiogenesis to extrapolate an association between these genes and different pathways involved in nm23 regulated metastasis. The differential expression of some of these genes from each group was confirmed by semi-quantitative RT-PCR. These results suggest that nm23 may down regulate the expression of certain cell adhesion and motility genes such as ErbB2, fibronectin 1, catenin, collagen type VIII alpha 1, TGF beta 2, FGF7, MMP14 and 16, and several integrins (Alpha 2, 8, 9, L and V) and tumor/metastasis suppressor genes such as PTEN and 2 members of SWI/SNF related matrix associated proteins (2 and 5). We used an in vivo spontaneous (mammary fat pad) metastasis SCID mouse model to confirm that overexpression of nm23 gene suppresses metastasis. We observed that the mice injected with nm23 overexpressing cells showed reduced lung metastasis as compared to those that were injected with low nm23 expressing cells. Thus our studies demonstrate that nm23 gene plays a major role in metastasis and its mechanism of action of metastasis suppression may involve down regulation of cell adhesion and motility genes and also certain important tumorigenesis/metastasis suppressors. Grant support from IH Klinger Research Foundation to HZ, US Army Medical Research Materiel Command (DAMD 17-99-1-9055) to MPA and National Institutes of Health (NCI CA 17613) to MJU are gratefully acknowledged.



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PANELISTS

MOHAN ACHARY, PH.D.

Dr. Mohan Achary is a cancer biologist and an assistant professor in the Department of Radiation Oncology at Albert Einstein College of Medicine, Bronx, NY. He did his Ph.D. on molecular mechanisms of DNA replication in India. He is presently funded by Radiation Therapy Oncology Group and the Department of Defense Breast Cancer Research Program to develop novel molecular markers for predicting the response of cervical carcinomas to specific types of therapy and for determining which mammary carcinomas will become metastatic. He teaches and supervises medical residents in the departments of Radiation Oncology and Molecular Genetics at Einstein.

REV. RAYMOND C. DEMPSEY

Rev. Raymond Dempsey serves as associate minister at Ebenezer Baptist Church in Virginia Beach. Rev. Dempsey received the Lord Jesus Christ as his personal savior in 1953 at the age of 8 years old and was ordained as a deacon at Community Baptist Church at 18. He was licensed to preach on Aug. 8, 1993.

He attended Marian College, where he obtained a B.S. in business administration in 1975 and completed 42 hours of an MBA program at Wichita State University in 1983. In May 2000 he received a certificate in religious studies from the Evans-Smith Leadership Training Institute, co-sponsored by The Samuel DeWitt Proctor School of Theology of Virginia Union University and the Baptist General Convention of Virginia.

He served in the U.S. Army from 1966-1978.

AMY E. GARBER

Amy E. Garber is a trial attorney with the Equal Employment Opportunity Commission's (EEOC) Norfolk Area Office, Norfolk, VA. She has been with the commission since September 1999 and is one of two attorneys in the Norfolk Area Office. In her capacity as trial attorney she represents the commission when it brings suit against private employers for violations of Title VII of the Civil Rights Act of 1964 (as amended), the Equal Pay Act, the Americans with Disabilities Act and the Age Discrimination in Employment Act.

Prior to joining the commission, Ms. Garber spent almost five years in private practice in Newport News representing employees and employers in employment related matters in federal and state courts and in administrative settings. She is a 1994 graduate of the University of Richmond's T.C. Williams School of Law.

At present she is also functioning half-time as an administrative judge for the commission.

MARK GRAY, PH.D.

Dr. Mark Gray, a professor in the department of biology chemistry and environmental science (BCES) at Christopher Newport University, is an instructor of microbiology, genetics and molecular biology. Dr. Gray earned a Ph.D. in pathology from Thomas Jefferson University in Philadelphia, PA. He continued his studies on the relationship between mechanism of DNA

CONFERENCE PROGRAM

8:30 a.m. Continental Breakfast
9 a.m. Registration
9:30 a.m. Greetings

Purpose of the conference–**Stevalynn Adams**

Honorable Yvonne B. Miller–Fifth Senatorial District, Virginia State Senate
Michelle Ellis Young–President, Beta Theta Zeta Chapter (Norfolk, VA), Zeta Phi Beta Sorority, Inc.

10 a.m. Introduction of Keynote Speakers

The Human Genome Project: From Inception to Present
Dr. Bettie J. Graham–Program Director, Division of Extramural Research
National Human Genome Research Institute (NHGRI)
National Institutes of Health (NIH), Bethesda, MD

The Human Genome Project:
Ethical, Legal and Social Issues of the Human Genome Project
Dr. Charmaine Royal–Principal Investigator, GenEthics Unit
National Human Genome Center, Howard University, Washington, DC

11:15 a.m. Break
11:30 a.m. Panel Discussion

Moderator:
Dr. Mark Gray–*Professor of Biology Chemistry and Environmental Science (BCES)*
Christopher Newport University, Newport News, VA

Panelists:
Dr. Mohan Achary–*Cancer Biologist and Assistant Professor*
Albert Einstein College of Medicine, Bronx, NY

Rev. Raymond C. Dempsey–*Associate Minister*
Ebenezer Baptist Church, Virginia Beach, VA

Amy E. Garber–*Trial Attorney*
Equal Employment Opportunity Commission's (EEOC)- Norfolk Area Office, Norfolk, VA

Dr. Debabrata Majumdar–*Professor of Biology*
Norfolk State University, Norfolk, VA

Dr. Virginia Proud–*Pediatrician and adult genetic counselor*
Children's Hospital of the King's Daughters (CHKD), Norfolk, VA

Dr. Hui Zhao–*Research Associate*
Albert Einstein College of Medicine, Bronx, NY

12:30 p.m. Complimentary Luncheon
1:15 p.m. Wrap up–What Next?
1:30 p.m. Presentations, special recognitions and closing

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91-033 - 475-3681 [O]
91-033 - 442-4678 [R]
Fax: 91-033-476-4419

Date.....

3- 15-2001

To
Dr. Mohan P. Achary
Assistant Professor and Cancer Biologist
Department of Radiation Oncology
Jack and Pearl Resnick Campus
1300 Morris Park Avenue
Bronx,
New York 10461
USA

Dear Dr. Achary :

I came to know from a reliable source that you will come to Kolkata, India, in April, 2001. If you can arrange your travel plans, you are welcome to visit our department, especially our laboratory in the Department of Zoology, University of Calcutta and you could also give a lecture to our students. I shall be highly obliged if you could accept the invitation and inform us if you could like to deliver a lecture on the topic chosen by yourself.

I am looking forward to hearing from you.

(Prof. R. N. Chatterjee)
Professor in Zoology
University of Calcutta

PHONE : 475-3681 (4 Lines)

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5- 15-2001

To whom it may Concern

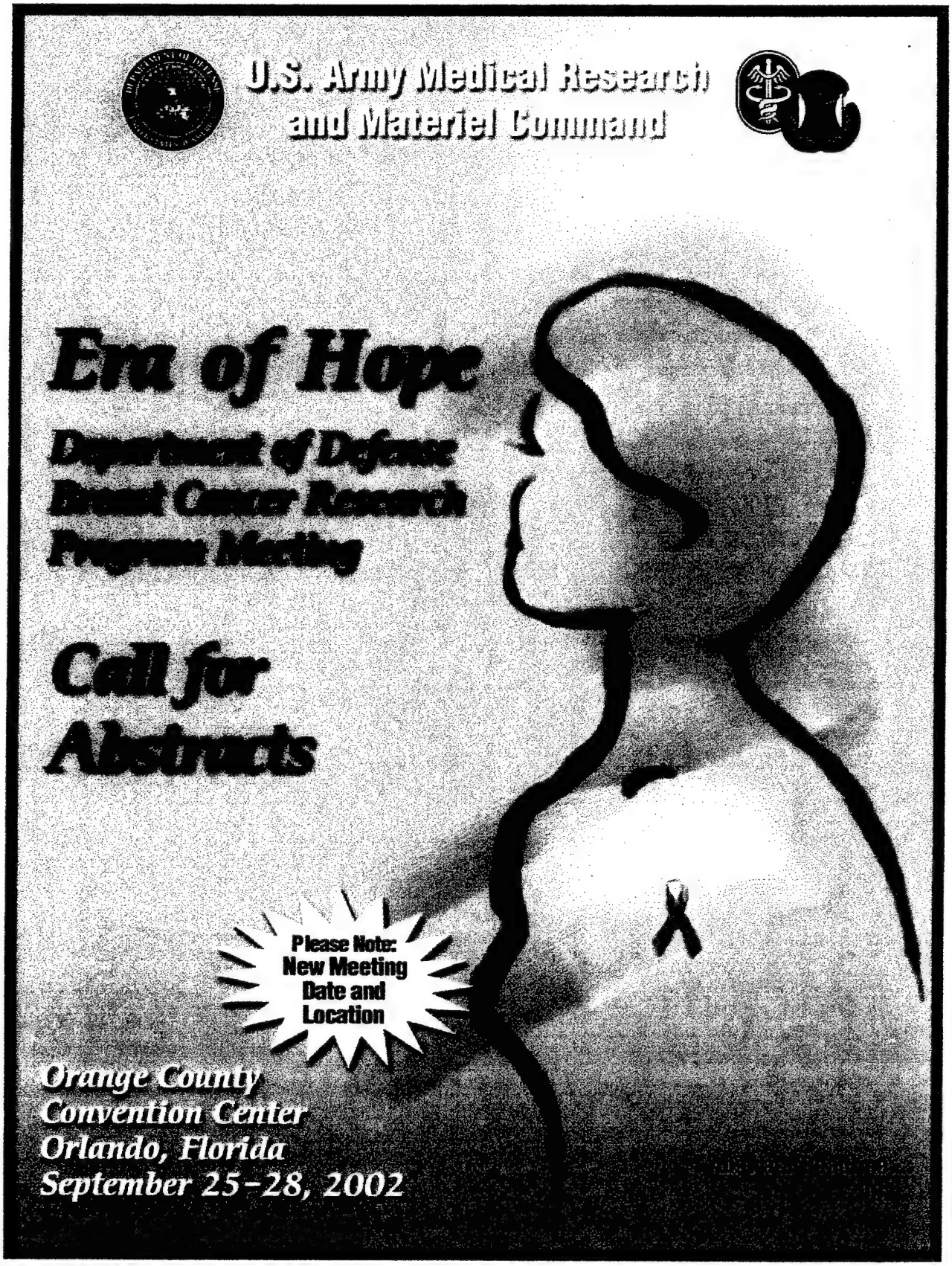
This is to certify that Dr. Mohan P. Achary, Ph.D. Assistant Professor and Cancer Biologist, Department of Radiation oncology, has delivered a lecture entitled " Molecular markers of breast metastesis and cervical carcinomas " in our department. Our students and a large number of research fellows have benifited from his deliver. I wish him every success in life.

Benarja
15.5.01
(Prof. B. Manna)

Head

Department of Zoology

Prof. & Head
Department of Zoology
University of Calcutta



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DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
1077 PATCHEL STREET
FORT DETRICK, MD 21702-5024

REPLY TO
ATTENTION OF:

Please note the following:

The abstract entitled: MOLECULAR MARKERS OF METASTASIS IN DUCTAL MAMMARY CARCINOMA

for contract: DAMD179919055

to be presented by: Patnala Achary

at the September 25–28, 2002, Era of Hope meeting will be presented in Poster Session: **Tumor Progression I, P9-1**

that is scheduled for: **Thursday, September 26, 2002, at 6:30-8:30 p.m.**

Poster Session Setup:
September 25, 2002, from 1:00 p.m. – 6:00 p.m.
September 26, 2002, from 7:00 a.m. – 3:00 p.m.

During designated Poster Session periods, Principal Investigators, or designated representatives, will be required to be at their posters to discuss their work.

Posters must be taken down by 10:00 a.m. on September 29, 2002.

MOLECULAR MAKERS OF METASTASIS IN DUCTAL MAMMARY CARCINOMA

P. Mohan R. Achary, H. Zhao, Z. Fan, H. P. Klinger[1] and B. Vikram

Metastasis Laboratory, Departments of Radiation Oncology and Molecular Genetics[1], Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY.

achary@aecom.yu.edu

About 13% mammary carcinoma patients with negative lymph nodes are prone to developing metastasis even if treated with surgery, or surgery and radiation therapy. The objective of this project is to construct a panel of molecular genetic markers for detecting those 13% of patients so that they could be treated more aggressively.

We hypothesize that in order to achieve the metastatic state primary mammary carcinoma cells must acquire genetic changes in addition to those that led to transformation. A subtractive DNA hybridization technique, Representational Difference Analysis (RDA) was used to compare the DNA of cells from archival normal tissue or primary ductal tumor with that of the metastatic lymph node of the same patient in order to isolate those sequences that were lost in the course of tumor metastasis. The tumor and metastatic cells were recovered by laser capture microdissection and subjected for RDA and also used for Southern blotting and PCR screening with the probes obtained from the RDA procedure.

We isolated 11 sequences that are candidates for being metastasis associated gene sequences (MAGS) because they were lost in metastatic cells. Radiation hybrid (RH) mapping of these sequences agreed with the results of sequence homology localization searches. To-date three of these 11 sequences were used to screen normal, primary and metastatic cell DNA samples. MAGS-XI was found to be lost in the metastatic cells of 3 out of the 5 tumors. MAGS-IX was found to be lost in metastases from 2 out of 3 primary tumors, and MAGS-IV was lost in 1 out of 3 tumors. RH mapping and homology search results indicated that MAGS-IX was located on the long arm of chromosome 10 where the PTEN, a known metastasis suppressor gene is also located. To determine if MAGS-IX is perhaps a part of the PTEN gene we PCR screened the above mentioned three tumor cell DNA samples and a breast carcinoma cell line, HCC-1937 which has homozygous loss of the PTEN gene (10q23 region). The results indicated that MAGS-IX is not related to the PTEN gene but is a novel gene sequence. Presently we are isolating partial and/or full-length sequences of these MAGS to use as fluorescence *in situ* hybridization (FISH) probes to screen a larger number of tumor samples. A 2Kb sized MAGS-IX has been generated and localized to the q21 region of human chromosome number 10 by FISH (Figure). Screening the primary tumor tissue sections of carcinomas which metastasize to lymph nodes is underway. The panel of molecular markers that we expect to develop should make it possible to detect those tumors (~13%) that are prone to becoming metastatic.

Fig. Localization of MAGS-IX to human metaphase chromosome 10q21 region by FISH. Centromere of chromosome 8 was used as a positive control probe. Both sequences were labeled with spectrum green (Vysis) and chromosomes were counter stained by DAPI.

The U.S. Army Medical Research Materiel Command under DAMD17-99-1-9055 supported this work.

FAX # (718) 430-2454

Lucio Luzzatto, MD
Courtney Steel Professor and Chairman
Department of Human Genetics

September 28, 1999

Dr. P.M. Achary
Dept of Radiation Oncology
and Department of Molecular Genetics
Albert Einstein College of Medicine
1300 Morris Park Avenue
Bronx, NY 10461-1802

Dear Dr. Achary:

Thank you very much for sending in a title for the forthcoming meeting of the New York Human Genetics Club. As you see, this is included in part I of the programme.

The time available for each presentation will be ***10 minutes***, plus ***5 minutes for questions***. I know this is a short time, but I hope that, by keeping the introduction to a minimum, you will find it still possible to present compellingly your main new findings.

To keep the programme layout simple, I have included only the name of the speaker. It is left to you to mention during your talk who is in charge of the lab, your collaborators, etc.

I am very much looking forward to seeing you on October 14th. Please kindly confirm with Valerie Charles at (212) 639-6165.

With best regards,

Lucio Luzzatto VAC

Lucio Luzzatto, M.D.

cc: Harold Klinger

Dictated but not read.

Memorial Sloan-Kettering Cancer Center
1275 York Avenue, New York, New York 10021
Telephone 212.639.6165 • FAX 212.717.3374
E-mail: l-luzzatto@ski.mskcc.org

The Eighth Meeting of the New York Human Genetics Club
October 14, 1999
The New York Blood Center
310 East 67th Street

PROGRAM

5:00 **The β-globin LCR controls expression levels but does not prevent hetero-cellular position effects** – Raouf Alami (E. Bouhassira), Albert Einstein College of Medicine

5:15 **Nipped-B, A drosophila homologue of chromosomal adherins, participates in activation by remote enhancers in the cut and ultrabithorax genes** – Rob Rollins (Laboratory of Dale Dorsett) – Memorial Sloan-Kettering Cancer Center

5:30 **Genotypic analysis of human β2-adrenergic receptor gene in long-QT syndrome and normal subjects** - Xiaoyan Qu (J. Russo/I.S. Edelman), Columbia Genome Center, Columbia University

5:45 **Gene expression profiles of cervical carcinoma cell lines with different degrees of radiosensitivity obtained with the cDNA microarray method** – P.M. Achary (Harold Klinger) – Albert Einstein College of Medicine

6:00 **Mechanism of double-strand break formation during meiotic recombination initiation in S. cerevisiae** –Scott Keeney – Memorial Sloan-Kettering Cancer Center

6:15 **Somatic imprinting of LCR containing cassettes in mouse erythroleukemia cells** – Yong-Qing Feng (E. Bouhassira), Albert Einstein College of Medicine

6:30 *TEA BREAK*

7:00 **Development of an antistathmin ribozyme-based strategy for gene therapy of cancer** - Sucharita J. Mistry (Laboratory of George Atweh) – Mt. Sinai School of Medicine

7:15 **Retroviral mediated transfer of the human glucose 6-phosphate dehydrogenase (HG6PD) gene rescues G6PD deficient phenotype of G6PD-null embryonic stem cells** – Olga Camacho Vanegas (Laboratory of Lucio Luzzatto), Memorial Sloan-Kettering Cancer Center

7:30 **PHRANC (a PML-HPRR retinoic acid-dependent nuclear complex) is essential for the tumor-growth suppressive activity of retinoic acid** – Sue Zhong (Laboratory of Pier Paolo Pandolfi), Memorial Sloan-Kettering Cancer Center

7:45 **Phenotypic consequences of a hypomorphic mutation in the mouse BRCA1 gene** – Thomas Ludwig, Columbia University

8:00 **Interphase FISH of polycythemia vera (PV): higher sensitivity and new insights into PV** – Lya Montella (A. Scalise, V. Najfeld/Tumor Cytogenetics), Mt. Sinai Medical Center

8:15 *BUFFET DINNER*

Prediction of Tumor Response to Therapy: Molecular Markers and The Microenvironment

Scientific Director: Dr. Shirley Lehnert
Radiation Oncology Division
McGill University

La prédiction de la réponse à la thérapie par la tumeur : les marqueurs moléculaires et le microenvironnement

Directeur scientifique : Dr Shirley Lehnert
Division de radio-oncologie
Université McGill

Holiday Inn Montreal Midtown
October 8-9 *octobre* 1999
Montréal, Canada

McGill

cDNA microarray gene expression analysis of cervical carcinoma cell lines with different degrees of radiosensitivity.

P. M. Achary, J. Wane, H. P. Klinger* and B. Vikram
Departments of Radiation Oncology and *Molecular Genetics, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY

The ability to simultaneously monitor expression of large number of genes presents major biological opportunities and exciting bioinformatic challenges. In the present investigation we used cDNA microarray chips to identify the differential expression of genes in human cervical carcinoma cell lines with different degrees of sensitivity to ionizing radiation. These microarray chips and the image scanning programs were recently developed in the *Genome microarray facility* of *Albert Einstein College of Medicine*. Each human cDNA microarray chip contains 5776 cDNAs representing several known genes, house keeping genes and ESTs. Fluorescent cDNA probes for hybridization were derived from radiosensitive HT137/5 (SF2= 0.28) and radioresistant HT180/1 (SF2= 0.70) cell lines. The cDNA samples from HT137/5 and HT180/1 were labeled with Cy3 (green) and Cy5 (red) fluors respectively. The fluorescence signals representing hybridization to each arrayed gene were analyzed using the *ScanAlyze2* software program to determine the relative abundance in the two samples of mRNAs corresponding to each gene. In the present investigation, significant differences in the expression of certain genes in these two cervical cell lines were observed. While radiosensitive cell line showed overexpression of *cyclin-dependent kinase 4 inhibitor*, *TGFβ 2*, *EGF receptor substrate*, *interferon _ receptor accessory factor* and several ESTs, the radioresistant cell line on the other hand showed expression of genes like *6-O methyl guanine DNA methyl transferase* (*MGMT*), *insulin like growth factor 1B* and several ESTs. Since *MGMT* gene is a DNA repair gene, its overexpression in the HT 180/1 cell line seems to be responsible for its radioresistant phenotype. Northern blotting studies are underway to confirm the overexpression of *MGMT* gene in HT180/1 cell line. Since *MGMT* was not previously implicated in radiation sensitivity/resistance, we also have extended our studies to the cDNA microarray gene expression profiles of these cervical cell lines with and without exposing them to ionizing radiation to further determine its role. These studies may prove valuable in determining the complex gene expression profiles for cytotoxic agents in a particular tumor thereby providing new leads for tumor diagnosis and intervention.

onstrated that the conjugate was stable in vivo. The result implies that F-18-RC160 may be a potential radiopharmaceutical for breast cancer detection by PET.

#4504 HUMANIZED ANTI-CEA X CHIMERIC ANTI-[INDIUM-DTPA] BISPECIFIC ANTIBODY CONJUGATES FOR RADIOIMMUNOTHERAPY OF CEA-POSITIVE TUMORS. Habibe Karacay, W. McBride, R. M Sharkey, H. Hansen, and D. M Goldenberg, *Golden State Cancer Ctr, Belleville, NJ, and Immunomedics, Inc, Morris Plains, NJ*

A two-step pretargeting approach using a bispecific antibody (bsMAb) followed by a radiolabeled peptide is described. As a prelude to constructing a fully humanized engineered bsMAb for such targeting, we have chemically prepared a series of human/chimeric bsMAb, hMN-14 x c734 (anti-carcinoembryonic antigen [CEA] x anti-indium-DTPA [In-DTPA]. To determine an optimal configuration for therapeutic applications; [IgG x Fab'], [F(ab')$_2$ x Fab'] and [Fab' x Fab'] conjugates were prepared. For imaging and therapy with Tc-99m and Re-188, respectively, a peptide IMP-192, with 2 DTPAs and a chelate for selectively binding Tc-99m/Re-188, was synthesized (IMP-192: [Ac-Lys(In-DTPA)-Tyr-Lys(In-DTPA)-Lys(TscG-Cys-)-NH$_2$], TscG=3-thiosemicarbazonylglyoxyl). In preliminary studies, mice bearing GW-39 human colonic cancer xenografts were injected with I-125 labeled bsMAb conjugates. [IgG x Fab'] showed the highest tumor uptake and retention was observed for at least 7 days. Thus, the [IgG x Fab'] might allow delivery of the highest dose of the Re-188 labeled peptide to the tumor for radioimmunotherapy. (Supported in part by Grants CA 81760 and 37895 from NIH.)

#4505 PROTEASOME INHIBITION ENHANCES RADIOSENSITIVITY OF HUMAN PANCREATIC CANCER CELLS *IN VITRO*. Michael A Houston, Rong Liu, Karin Abendroth, Albert S Baldwin, and James C Cusack, *Univ of North Carolina at Chapel Hill, Chapel Hill, NC*

Activation of NF-κB explains one mechanism of inducible radioresistance in a number of human cancer cell lines. Irradiation has been shown to induce activation of NF-κB in fibrosarcoma, colorectal, and pancreatic cancer cells. We have previously demonstrated that the proteasome inhibitor, PS-341 blocks NF-κB induction by chemotherapy in human pancreatic cancer cells. This study investigates the effect of proteasome inhibition on growth rate and survival in pancreatic cancer cells treated with γ-irradiation. Human pancreatic cancer cells (BxPC-3, CFPAC, and Panc-1) were grown in 6-well plates at 37° C at 5% CO_2. Cells were treated with PS-341 (1 μmol) diluted in growth medium, or growth medium alone for one hour. Three hours later, cells were irradiated at 6Gy or 8Gy using a ^{137}Cs source. Controls were sham-irradiated and the medium was replaced for all cells following irradiation. Treatment groups were in triplicate wells/day, and included γ-irradiation only, PS-341 + γ, PS-341 only, and negative controls. Cell counts were performed for days 0-3. For all cell lines, treatment with PS-341 or 6Gy alone resulted in a minimally decreased growth rate compared to controls. Combined treatment with PS-341 and 6Gy resulted in a statistically significant reduction in cell number at day 3 ($p<0.001$, ANOVA) for CFPAC and BxPC-3 cells, but not for Panc-1 cells ($p<0.06$, ANOVA). Panc-1 cells showed a significant difference when treated with PS-341 + 8Gy ($p<0.002$, ANOVA). These findings suggest that proteasome inhibition with PS-341 may play a role in enhancing the radiosensitivity of pancreatic cancer cells. Studies are underway to further characterize this effect, and to determine the effect of PS-341 on NF-κB induction by irradiation in pancreatic cancer cells.

#4506 DIFFERENTIAL GENE EXPRESSION PROFILES IN RADIORESISTANT CERVICAL CELL LINES BY CDNA MICROARRAYS. Mohan P Achary, Jaggernauth Wainwright, Eugene Gross, Rafik Khaimov, Harold P Klinger, and Bhadrasain Vikram, *Albert Einstein Coll of Medicine, Bronx, NY*

We hypothesize that the underexpression or overexpression of specific genes is responsible for the ability of a cervical carcinoma cell to become radioresistant. To identify differential expression of genes in radioresistant cervical carcinoma cell lines, we initially performed cDNA microarray analysis using radioresistant HT180/1 (SF2= 0.70) and radiosensitive HT 137/5 (SF2= 0.28) cell lines derived from two different patients. This study (Experiment I) identified about 105 overexpressed and 93 underexpressed genes. Subsequently, we have undertaken similar experiments but used two pairs of cell lines, each pair derived from a single patient tumor (in order to avoid genetic variations between individuals). Analysis of the first pair of cell lines [HT180/1 (SF2= 0.7) vs. HT180/8 (SF2= 0.34)] showed overexpression of 11 known genes and underexpression of 16 known genes (Experiment II). In the second pair of cell lines [HT137/8 (SF2= 0.48) vs. HT137/5 (SF2= 0.28)], 39 known genes were overexpressed and 9 known genes were underexpressed in the radioresistant cell line (Experiment III). Super oxide dismutase was found overexpressed in experiments I and III. Cytochrome oxidase-C, p53 and ribosomal protein were found overexpressed in experiments I and II. Northern blot experiments are being performed to verify the expression levels obtained for these genes in microarray experiments.

#4507 MULTIDRUG RESISTANCE (MDR) AND RADIATION RESISTANCE IN HUMAN SMALL CELL LUNG CANCER CELLS TREATED WITH FRACTIONATED RADIATION. Rozelle M Harvie, Hai Ping Sun, Sheridan Henness, Greg B Peters, Mary W Davey, and Ross A Davey, *Royal North Shore Hosp, Sydney, Australia, and Univ of Technology, Sydney, Sydney, Australia*

Small cell lung cancer (SCLC) is typical of cancers that initially respond well to chemotherapy and radiotherapy but subsequently develop drug and radiation resistance. Despite the clinical evidence for an association between drug and radiation resistance, there are few cellular models in which to study this relationship and ways of circumventing resistance. We therefore treated the H69 "classic" and H82 "variant" human SCLC cell lines with fractionated radiation (X-rays) consisting of a total dose of 37.5 Gy given over 30 weeks to produce the H69/R38 and H82/R38 sublines. The H82 cells were inherently more radiation resistant with higher levels of Multidrug Resistance-associated Protein (MRP) than the H69 cells. The H82/R38 subline showed little increased resistance to radiation but had developed low level resistance to cisplatin, the anthrcyclines, gemcitabine and taxol. In contrast, the H69/R38 subline was over 2-fold radiation resistant, 12-fold resistant to cisplatin, 8-fold to daunorubicin, 15-fold to epirubicin, 5-fold to gemcitabine but was not resistant to taxol. This was accompanied by a small increase in MRP expression, no significant change in either Lung Resistance Protein (LRP), bcl-2 or p53 expression, and P-glycoprotein was not detected in either subline or in the parental cells. Cytogenetic analysis of the H69/R38 subline showed several rearrangements, but none of these have been associated specifically with MDR. These sublines with radiation-induced radiation resistance and MDR therefore provide cellular models in which the molecular mechanisms and the cellular response pathways involved can be defined.

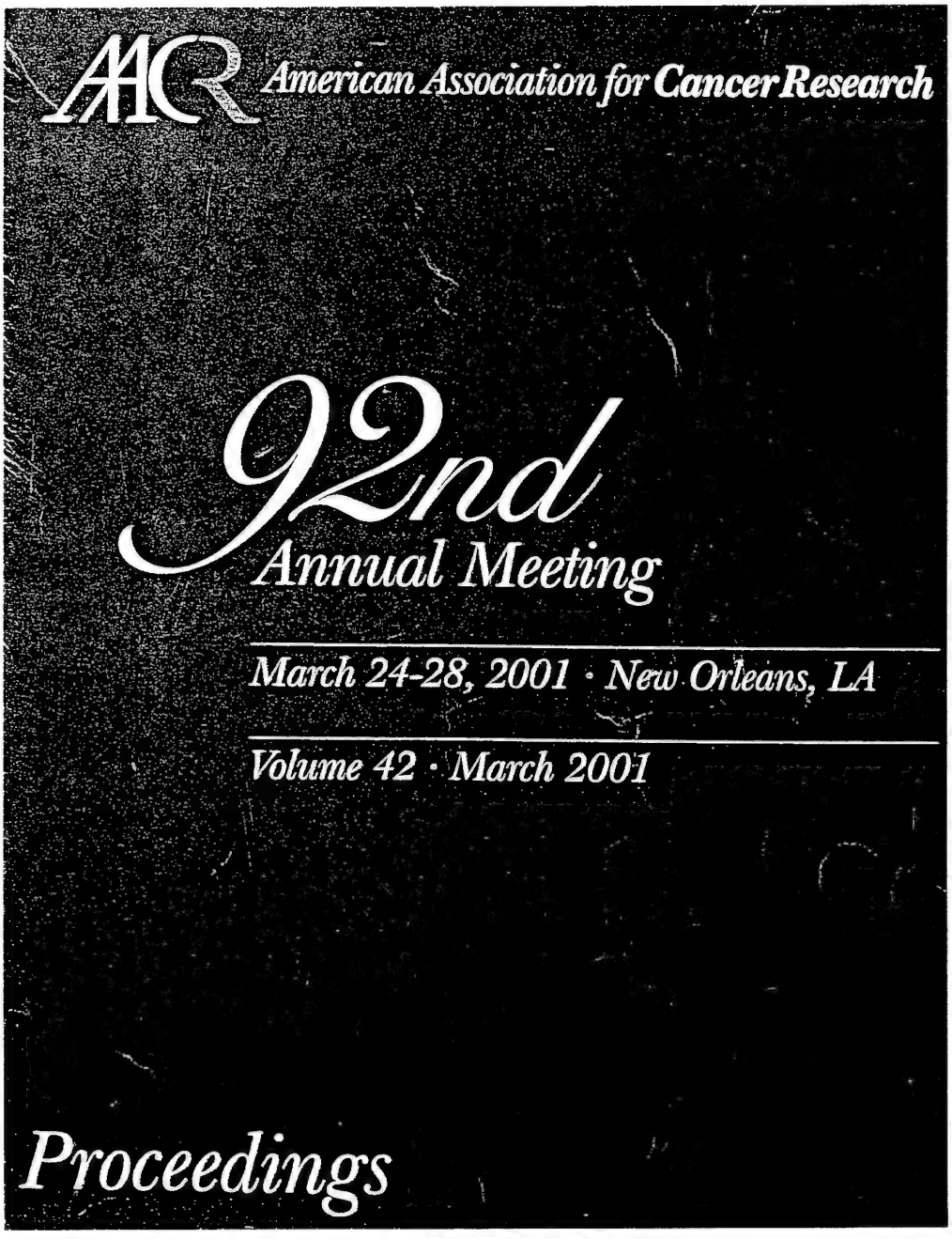
#4508 IN VIVO ENHANCEMENT OF TUMOR RADIORESPONSE BY C225 ANTI-EPIDERMAL GROWTH FACTOR RECEPTOR ANTIBODY. Luka Milas, Kathy Mason, Nancy Hunter, Sven Petersen, Michitaka Yamakawa, Kian Ang, John Mendelsohn, and Zhen Fan, *U T M D Anderson, Houston, TX, and U T M D Anderson Cancer Ctr, Houston, TX*

Overexpression of epidermal growth factor receptor (EGFR) has been correlated with tumor resistance to cytotoxic agents, including radiation (Akimoto et al., Clin. Cancer Res., 5:2884, 1999), and thus is a candidate target for anticancer treatment. This study investigated whether treatment with C225 anti-EGFR antibody would improve tumor response to radiotherapy. Nude mice bearing 8 mm diameter A431 tumor xenografts in the hind leg were treated with C225 antibody, 18 Gy of single-dose local tumor irradiation, or both. C225 was given i.p. at a dose of 1 mg/mouse 6 h before irradiation or 6 h before and 3 and 6 days after irradiation. Delay in tumor growth was the treatment endpoint. C225 dramatically improved the efficacy of local tumor irradiation, particularly when multiple injections of C225 were administered. Tumor radioresponse was enhanced by a factor of 1.59 by a single dose and by a factor of 3.62 by 3 doses of C225. Histological analyses of tumors revealed that C225 caused a striking increase in central tumor necrosis associated with hemorrhage and vascular thrombosis when combined with radiotherapy. In addition, C225 induced heavy tumor infiltration with granulocytes, increased tumor cell terminal differentiation, and inhibited tumor angiogenesis. We conclude that C225 anti-EGFR antibody enhances tumor radioresponse by multiple mechanisms that may involve direct and indirect actions on tumor cell survival.

IMMUNOLOGY/PRECLINICAL AND CLINICAL 15: Tumor Antigens and Restricting Elements

#4509 EXPRESSION OF THE TUMOR-REJECTION ANTIGEN SART3 IN COLORECTAL CANCERS. Teruo Sasatomi, Damu Yang, Yoshiaki Miyagi, Hideaki Yanama, Yutaka Ogata, Kazuo Shirouzu, and Kyogo Itoh, *Kurume Univ Sch of Medicine, Kurume, Fukuoka, Japan*

We recently reported the tumor-rejection antigen, SART3, (Squamous cell Antigen Recognized by T cells-3) that possesses tumor epitopes capable of inducing cytotoxic T lymphocytes (CTLs) in epithelial cancer patients. This study investigated the expression of SART3 antigen in colorectal cancers to explore for a potential molecule for use in specific immunotherapy of patients with colorectal cancers. The SART3 antigen was investigated by Western blotting in all 6 colorectal cancer cell lines and in 37 colorectal cancer tissues. PBMCs of the HLA-A24+ colorectal cancer patients were stimulated by SART3 derived peptides. The SART3 antigen was detected in the cytosol and nuclear fraction of all 6 colorectal cancer cell lines and in 27 of 39 (69%) colorectal cancer tissues. The HLA-A24 restricted and SART3 derived peptides induced CTLs recognized the HLA-A24+ and SART3+ colorectal cancer cells. Therefore, the SART3 antigen could be a target molecule for specific immunotherapy of patients with colorectal cancers expressing HLA-class I antigens. Since October 1999, we have tried the phase 1 clinical trial of this cancer vaccine for the HLA-A24+ colorectal patients.


AACR
American Association for Cancer Research
92nd
Annual Meeting
March 24-28, 2001 · New Orleans, LA
Volume 42 · March 2001
Proceedings

#623 Differential Gene Expression Associated with Tumorigenicity of Cultured Green Turtle Fibropapilloma-Derived Fibroblasts. Lawrence H. Herbst, Ratna Chakrabarty, Paul A. Klein, and Mohan P. Achary. *Albert Einstein College of Medicine, Bronx, NY, Department of Radiation Oncology, Albert Einstein College of Medicine, Bronx, NY, University of Central Florida, Gainesville, FL, and University of Florida, Gainesville, FL.*

Fibropapillomatosis (FP) is a devastating neoplastic disease of green sea turtles (Chelonia mydas) and other endangered sea turtle species. This study was an initial attempt to apply functional genomics to elucidate the etiopathogenesis of this disease. Fibroblast cell lines derived from normal skin and experimentally induced tumors taken from the same individual turtle were tested for tumorigenicity in immunodeficient mice. Differential Display RT-PCR was used to identify differences in messenger RNA expression between normal cells and tumorigenic FP cells. Four unique products that were apparently overexpressed in FP and three that were apparently underexpressed were cloned and sequenced. Differential expression was confirmed for three products by Northern blotting. Two overexpressed products showed extensive sequence matches to the known mammalian cellular genes, β−hexosaminidase (HEXB) and chain termination factor (HCGVII). The product that was underexpressed in FP showed homology with mammalian thrombospondin (TSP-1), a known tumor-suppressor gene and an inhibitor of angiogenesis. All of the partial gene sequences identified are novel and will require full-length cDNA sequencing to further characterize them. These results however provide the foundation for further investigation to determine the role of each of these gene products in FP pathogenesis and cellular transformation. The potential for some of these products to serve as biomarkers for FP is discussed.

#4937 Involvement of p53 and BRCA1 genes in breast cancer in African-American and white women. Meena Jhanwar-Uniyal, Gina Day Stephenson, Renee Royak-Schaler, Chung-Xiou Wang, Mohanrao Achary, Anthony P. Albino, and John Whysner. *Institute for Cancer Prevention (American Health Foundation-Cancer Center), Valhalla, NY and Albert Einstein College Of Medicine and Montefiore Medical Center, Bronx, NY.*

The p53 and BRCA1 tumor suppressor genes play a key role in establishing genome stability. Inactivation or mutation of p53 is seen in variety of cancers including breast cancers. There have been discrete p53 mutations in tumors from African-Americans (AA) that were different from those observed in White Americans (WA). Germline mutations in BRCA1 have been reported in hereditary breast cancer, but somatic mutations of BRCA1 gene are absent in sporadic cancers. Recent studies have shown that inactivation of BRCA1 occurs via the hypermethylation of the promoter region of the BRCA1 gene. The purpose of this study was to clarify the role of two susceptibility genes as determinants or potential modifiers of outcome differences in African-American and White women diagnosed with breast cancer. AA breast cancer patients more frequently have clinical and pathological features of advanced disease and reduced survival than their white counterparts. Well-characterized tumor tissues from 94 breast cancer patients (34 AA and 60 WA) diagnosed at two Philadelphia hospitals were screened for mutations of p53 and BRCA1 inactivation using various methods including immunohistochemistry, DNA-modification followed by methylation-specific PCR, PCR-SSCP analyses and a direct DNA sequencing. Our results show that: 1) the number of p53 negative cases were lower in AAs as compared to WAs, 2) p53 overexpression of mutant protein was more commonly observed in AAs than WAs, and also the number of p53 mutations were greater in AAs compared to WAs, 3) hypermethylation of promoter of BRCA1 gene occurred in cases where p53 was mutated with concurrent negative estrogen receptor (ER)/progesterone receptor (PR) status in both the AA and WA tumors. No BRCA1 inactivation was detected among the patients without p53 mutations or in those with p53 mutations and ER/PR positive tumors. We hypothesize that loss of expression of ER and PR proteins leads to genomic instability that may result from the inactivation of p53 via mutation and BRCA1 via hypermethylation. Data from this study suggest that variation in these genetic susceptibility factors may have prognostic significance in AA and WA breast cancer patients. (Supported by NCI CA 17613, DAMD-17-99-1-9055 (M.A.), DAMD-17-00-1-0675 (M.J-U.))

Annexure VIII

ALBERT EINSTEIN COLLEGE OF MEDICINE
OF YESHIVA UNIVERSITY
1300 MORRIS PARK AVENUE • BRONX, NEW YORK 10461

HAROLD P. KLINGER MD, PhD
DEPARTMENT OF MOLECULAR GENETICS

PHONE: (718) 430-2451
FAX: (718) 430-2454
EMAIL: hklinger@aecom.yu.edu

March, 1 2001

Dr. Mohan Achary
Department of Radiation Oncology
Albert Einstein College of Medicine
Bronx, New York

Dear Dr. Achary,

I am pleased to inform you that the Advisory Committee of the ***Iris and Harold P. Klinger Fund for a Postdoctoral Fellowship Stipend in Molecular Genetics*** has approved your application requesting the salary support of the Research Associate, Dr. Hui Zhao, to work on your projects for the period of one year. This support may be renewed based on the satisfactory progress of the proposed work.

Sincerely yours,

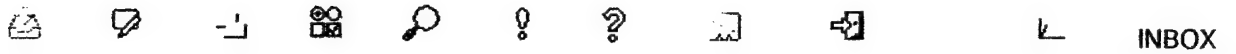
Harold P. Klinger MD, PhD
Professor of Molecular Genetics

Copies to:

1. Dr. B. Vikram, Chairman, Dept. of Radiation Oncology, AECOM/MMC.
2. Dr. R. Kucherlapati, Chairman, Dept. of Molecular Genetics, AECOM.
3. The Advisory Committee of IHPK Fund, NY.

HPK:bg

Annexure IX



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Date: Thu, 29 May 2003 12:06:08 -0400
From:
To:
Reply-to:
Subject: DOD FY03 Breast Cancer Research Program

Dear Dr. Achary

Your proposal entitled

Isolation and characterization of novel candidategenes associated with metastasis in ductal mammary
carcinoma

and sent to the U.S. Army Medical Research
and Materiel Command DOD 2003 Breast Cancer
Research Program has been received on time.

Your proposal # BC031993

should be used in all future Correspondence
concerning your proposal.

You may expect to hear from us regarding the
funding status of your proposal in
December, 2003, after the Programmatic
Review process is complete.

Thank you for your interest.

Kenneth A. Bertram, M.D., Ph.D., F.A.C.P.
Colonel, U.S. Army Medical Corps
Director, Congressionally Directed
Medical Research Programs

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This message to

Annexure X

MORRIS ANIMAL FOUNDATION
Est. 1948

July 10, 2002

Lawerence H. Herbst, DVM, PhD
Albert Einstein College of Medicine
Department of Pathology
1300 Morris Park Ave.
Bronx, NY 10461

Re: D02ZO-109, Albert Einstein College of Medicine
'The Viral Pathogenesis of Marine Turtle Fibropapillomatosis'

Dear Dr. Herbst:

I am pleased to inform you that the Wildlife Scientific Advisory Board (WSAB) of Morris Animal Foundation has recommended the approval of the above referenced proposal and the Foundation's Executive Committee has voted to fund it, according to the proposed budget. A critique is enclosed. **Please review it** and notify me, no later than August 1, 2002, if you plan to alter your original proposal, based on any comments or suggestions stated in the critique. In addition, any future change in key personnel, objectives, or protocol during the study must be submitted to the Foundation for **prior approval**..

Contracts are presently being written for the project and will be sent to the appropriate persons at your institution for signature. A copy will be forwarded to you as well. You can expect a start date of September 1, 2002.

The Foundation's policy is to withhold 50% of the last quarterly payment until we receive the final report of each grant. Our hopes are to encourage timely submission of final reports and alleviate cumbersome administrative activity.

We are pleased to have this opportunity to play a part in your on-going contributions to veterinary medicine and providing a healthier tomorrow for animals.

Congratulations, and I will be back in touch with you soon. Please fill out the enclosed postcard and return it as soon as possible.

Sincerely,

Kristin Benjamin
Director of Grants

KB/tlj

Encls.

MORRIS ANIMAL FOUNDATION PROPOSAL EVALUATION FORM FOR WILDLIFE SCIENTIFIC ADVISORY BOARD

1. School, title, investigator(s)
2. Brief summary of proposed research
3. Relevance to research interests of MAF & species studied
4. Critique of scientific merit
   A. Pertinence of literature review
   B. Conciseness of objectives
   C. Attainability of objectives
   D. Adequacy of experimental design
   E. Appropriateness of data analysis
   F. Additional pertinent comments
   G. If pilot study: potential to support more definitive study
5. Adequacy of staff and facilities
6. Adequacy of humane considerations (should IACUC be required?)
7. Appropriateness of budget
8. Overall assessment of scientific merit
9. Is this a resubmission?
10. Is this a continuation of a previous or current MAF funded study?

Proposal # D02ZO-10

(Begin here with item #1)

1. **School, title, investigator(s):** Albert Einstein College of Medicine, The Viral Pathogenesis of Marine Turtle Fibropapillomatosis; Lawrence H. Herbst, Jack Lenz, Mohan Achary

2. Brief summary of proposed research: A novel herpesvirus of green sea turtles is associated with the presence of fibropapillomas in a variety of marine turtles. Fibropapillomatosis (FP) neoplastic condition, found worldwide, and has serious implications for the recovery of threatened marine turtle species. The herpesvirus (GT-FPHV) associated with FP has not been cultured and there may be other viral agents or oncogenes that are responsible for cell transformation in the host. This proposal will complete the cloning, sequencing, and characterization of GT-FPHV, the primary candidate causing FP. By determining the complete genome sequence, it may identify potential viral oncogenes responsible for the development of neoplasia which is one of the specific aims. In addition, it will facilitate the development of diagnostic tests and vaccines which is another specific aim. Preliminary data comparing tumor versus normal DNA and RNA from green turtles using differential display and representational difference analyses have provided additional sequences that may be involved in FP. They may be other viral or host genes. These will be evaluated to determine if they are expressed in FP tumors and not normal tissues.

3. Relevance to research interests of MAF & species studied: Prevention/epidemiology of disease; wildlife/special species

4. Critique of scientific merit

A. Pertinence of literature review: The literature review defines the problem in historical terms and describes what has been done to identify the etiology. Much of this work has been done by Dr. Herbst, the PI. The background on FP is presented objectively and without bias.

B. Conciseness of objectives: The objectives are well explained and easy to follow.

C. Attainability of objectives: Between the pre-proposal and full proposal, progress has been made on detecting multiple entry points into the GT-FPHV genome and obtaining additional sequences using inverse-PCR. Work is moving quickly and it is expected that Aim A (complete sequence of GT-FPHV), Aim B (identification of other potential viruses or endogenous oncogenes) and Aim C (possible mechanisms of tumor formation) will be accomplished in a timely manner. Aim D (expression of selected viral genes *in vitro* to produce recombinant antigens for use in developing diagnostic assays and a vaccine) may take longer than the 3 year period requested for funding, but should be ultimately attainable.

D. Adequacy of experimental design: The experimental design optimizes the likelihood of detecting other putative viruses and host genes that may play a role in the development of FP. Preliminary data supports that the methods outlined are appropriate and relatively fast and efficient.

E. Appropriateness of data analysis: Data analysis relates to genomic analysis of viral sequences using well accepted computer programs.

F. Additional pertinent comments: None.

G. If pilot study: potential to support more definitive study: Not applicable.

5. Adequacy of staff and facilities: The PI, Dr. Herbst, is well-recognized for his work on the viral pathogenesis of FP in green turtles. He has established FP infected and uninfected green turtle fibroblasts and developed the mouse tumorigenecity assay used in this study. The co-investigators have considerable experience with the molecular techniques described in the proposal and have a solid background in viral oncogenesis. An experienced technician will devote 50% effort to this study for three years. All facilities and equipment needed to conduct this study are present at Albert Einstein College of Medicine.

6. Adequacy of humane considerations
(should IACUC be required?) An IACUC has been submitted to cover use of animals in this study. The PI holds the appropriate state and federal permits to conduct the turtle studies.

7. Appropriateness of budget: The budget is appropriate and well-justified. It is reassuring that a medical school would sign off on this proposal when full indirect costs cannot be recovered.

8. Overall assessment of scientific merit: This proposal has the potential to delineate the pathogenesis of FP in marine turtles and provide antigens for the development of diagnostic assays and vaccines. Probability of success is quite good.

9. Is this a resubmission? No

10. Is this a continuation of a previous or current MAF funded study? No

Cytogenetics and Cell Genetics

Founded 1962 as 'Cytogenetics' by H.P. Klinger

KARGER

Printed in Switzerland Appearance:

International Cytogenetics and Genome Society

A Call for MEMBERS of this new Society

The Editors of *Cytogenetic and Genome Research* and the founding members* of this Society perceived a need for vertebrate, invertebrate and plant cytogeneticists from all parts of the world to have their own international society. To determine if others shared this view they conducted a survey of established and respected investigators in the field. The result was that 74% of the responders found there was a need for such a Society and many expressed a high level of enthusiasm for the proposal. Consequently the *International Cytogenetics and Genome Society (ICGS)* was established in June of 2002.

Main objectives and functions of *ICGS*

1. Foster the exchange of knowledge and collaboration between investigators in plant, invertebrate, and vertebrate cytogenetics from different parts of the world by organizing international conferences and by providing Internet facilities for such exchanges.

2. Establish an organization that will give cytogeneticists greater visibility; represent them in various ways; and demonstrate that cytogenetics is still a very vigorous and important field.

3. Foster the *'ONE WORLD'* concept by attempting to counteract the propaganda that is disseminated in some countries in respect to other nations. This will be done by promoting interaction of investigators of different countries and by promoting training fellowship opportunities in two ways:

(a) Provide postdoctoral stipends by establishing an endowed fund to support postdoctoral trainees from developing nations.

(b) Encourage and provide the mechanism for Society members to enlist and support graduate and postdoctoral trainees in their laboratories.

4. Provide its members with very sizable discounts for subscriptions and publication costs for *Cytogenetic and Genome Research* and for other S. Karger journals and books (see below for details). In addition, we have also negotiated sizable rate reductions with other publishers for their publications and services. See the *(ICGS)* Internet site for details.

Benefits of Membership in *ICGS*

Members will be entitled to:

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A **25%** reduction of the registration fees for the International meetings of the Society.

A **25%** reduction in the processing fee, page charges, color plate fees and reprint costs of papers published in ***Cytogenetic and Genome Research (CGR).***

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* See ICGS home page for list of 90 founding members

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Register on-line at: http://cytserver.aecom.yu.edu/ccg/icgs/icgs__form.asp.

You can also register by completing the attached Membership Application Form and mailing it to the address given below.

Further information about the Society and the details of membership benefits can be found at: http://cytserver.aecom.yu.edu/ccg/icgs/icgs_info.htm

The various activities of the Society; listings of plans for the international conferences; listings of other meetings and events of interest to cytogeneticists will be posted here.

Advertisements for available positions will also be posted as well as those of individuals seeking employment. The latter may advertise at no charge. All advertisements should be sent to: hklinger@ aecom.yu.edu

We will be happy to answer any questions you may have about ***ICGS*** and will also appreciate any suggestions you may have.

We very much hope you will join ***ICGS*** and also encourage other colleagues and students to join. On the basis of responses we have received from many colleagues we believe it will become an intellectually stimulating organization from which its members will derive considerable benefits.

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